# CHROMOSOME STUDIES IN HUMAN LYMPHOCYTES

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In accordance with the regulations of the University of Edinburgh, I declare that I have composed this Thesis myself and that I carried out all the research described except where otherwise indicated.

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## ABSTRACT

### PART I. STUDIES USING INCORPORATION OF THE BASE ANALOGUE 5-BROMODEOXYURIDINE

New high resolution staining techniques for detection of BrdU-substituted chromatin have been applied to human chromosomes in investigations of their behaviour and structure.

A. Late Replication Studies. Giemsa staining using the FPG technique after incorporation of BrdU during the later stages of DNA synthesis was used to highlight the regions of the chromosomes that undergo late DNA replication and proved to be a particularly efficient method of detection of the 'inactive' X chromosome in female cells.

B. Asymmetrical C-Bands. After one complete cell cycle of BrdU incorporation, lateral asymmetry of staining by the FPG technique is visible in the major C-band regions of the karyotype. The implications of this for underlying base composition of the DNA and structure of chromatin are discussed.

C. Chromosome structure and behaviour. After two cell cycles in the presence of BrdU, there is pronounced differentiation in staining intensity between sister chromatids containing unequal amounts of the analogue. Exchanges between sister chromatids (SCE) are clearly visible.

Investigations on these 'Harlequin-stained' chromosomes showed random segregation of chromatids at mitosis and gave evidence that these chromosomes are unineme, with sister chromatids containing subunits of opposite polarities, and suggested that there may be a low level of spontaneous SCE. The distribution of SCE within and between chromosomes was examined in detail.

D. SCE, Chromosome Aberrations and Repair. SCE frequencies were examined in cells from individuals from all age groups and in several situations where there is evidence for deficiencies in mechanisms for repair of damage to DNA. These include people with constitutional chromosome abnormalities, the chromosome instability syndromes Ataxia Telangiectasia (AT) and Fanconi's Anaemia, and the UV-sensitivity disease Xeroderma Pigmentosum. No significant deviations from normal SCE frequencies were found in any of these individuals, although there was a consistent slight depression in SCE levels in cells from AT patients. The effect of X-radiation on SCE levels both *in vitro* and *in vivo* was tested, and although the frequency is increased after *in vitro* X-ray exposure, it is not a sensitive indicator of radiation damage. Chromosome and chromatid aberrations were also scored in these experiments, and although AT cells are hypersensitive to aberration induction by X-rays, their SCE levels were found to be normal. SCE levels are affected much more dramatically by chemical mutagens and I found that repair deficient AT cells were able to respond normally, with high levels of SCEs, to Mitomycin C, Ethyl Methane Sulphonate and Adriamycin. It was concluded

that SCEs are not directly linked to aberration formation and are not an indicator of any one known repair mechanism.

## PART II. STUDIES ON CHROMOSOME CHANGES WITH AGEING

Chromosome analysis using G-banding was carried out on cells from 65 males and 102 females of all ages, from a random sample of the population. The frequency of aneuploid cells showed a significant increase with age in both sexes, and in females the increase in hypodiploidy and hyperdiploidy was more marked than in males, and involved a high proportion of cells that had lost or gained an X chromosome, 45X cells being much more common than 47XXX cells. In females the occurrence of a 'fragment' of an X chromosome also correlated with increasing age, and this 'fragment' appears to be an X chromosome that has simply divided prematurely at the centromere. The effects of time in culture on proportions of abnormal cells of various types was also investigated, and the results are discussed in the light of findings from several other 'ageing surveys'.

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In conclusion, I would like to say how much I have enjoyed my time in this Unit, where I have made very many friends, besides having the opportunity to begin to learn something about research.

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GENERAL INTRODUCTION

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# GENERAL INTRODUCTION

The central problems in this work involve studies on several aspects of the structure and function of human chromosomes, including the use of some important new staining techniques to investigate normal age-related karyotypic variation, and also the characteristics of chromosomes in abnormal situations where aberrations occur either spontaneously, as in the 'chromosome breakage syndromes', or after exposure to mutagens. The new cytological methods allow not only the identification of each pair of human chromosomes, but also differentiation between the sister chromatids of single chromosomes, so that exchanges between these chromatids (SCE's) can be seen.

All this has become possible only since the beginning of this decade, and it is worth remembering that human cytogenetics is a very new science since it was 1956 before it was finally established that the number of chromosomes in normal human somatic cells was 46 (Tjio and Levan, 1956). Although the study of plant cytogenetics using 'squash' preparations of dividing tissues had been developing since the turn of the century, animal studies were hampered by difficulties in obtaining good chromosome preparations from meiotic or mitotic cells. The two major problems were the paucity of dividing cells, and the fact that the chromosomes of many animal species of interest, including man, are small, numerous and difficult to disperse or 'spread'. These obstacles were largely overcome by the development of tissue

culture techniques that provided adequate numbers of dividing cells, and the use of spindle inhibitors and hypotonic pretreatment (see, for example, Hsu, 1952; Ford et al., 1958) to facilitate spreading of the chromosomes in metaphase cells. However, even after relatively simple methods for culturing human blood lymphocytes (Moorhead et al., 1960) became widespread so that large numbers of mitotic cells could be examined, human chromosomes could not be identified individually apart from the distinctive pairs 1, 2, 3, 16 and often the Y chromosome, and were simply assigned to groups according to size and the relative proportions of arm lengths. The development of methods for distinguishing each chromosome pair has an interesting history.

# A. CHROMOSOME IDENTIFICATION

## i. Heterochromatin and Late DNA Replication

For decades cytologists have known that chromosomes are not uniform and featureless in their appearance, but have certain areas that are conspicuous due to irregularities of shape or staining intensity. As early as 1928 Heitz showed that in meiotic plant cells there were two types of chromatin that differed in their degree of condensation during interphase, though both types were highly condensed at metaphase (reviewed by Hsu, 1973). Heitz gave the name heterochromatin to the material that was densely compacted throughout the cell cycle, and euchromatin to the remainder of the complement that was decondensed during interphase. Studies on Drosophila revealed that in this species heterochromatin was genetically inert (Muller and Painter, 1932; cited by Comings, 1972), and the same correlation was shown to hold in normal female mammalian cells where the highly condensed sex chromatin seen in interphase cells (the Barr body, Barr, 1959) was shown to represent a single X chromosome (Ohno et al., 1959) and this X chromosome was genetically inactive (Lyon, 1961). Lyon originally proposed that the process of heterochromatinisation and inactivation of one of the X chromosomes was random with respect to the origin and gene content of the X chromosome - a proposal which has since received much support (reviewed by Lyon, 1968). This class of heterochromatin, which becomes heterochromatic during development, is generally referred to as facultative heterochromatin in

contrast to the more permanent constitutive heterochromatin (Brown, 1966) that appears to be devoid of DNA sequences coding for functional proteins.

Heterochromatin and its distribution was of little help in distinguishing human chromosomes until the discovery of an important property of this material, namely the characteristically late timing of DNA replication compared with the euchromatin, first discovered by Lima-de-Faria (1959) in autoradiographic studies on grasshoppers injected with tritiated thymidine. Taylor used similar tritium-labelling methods to demonstrate the same phenomenon in Chinese hamster cells treated with isotope during the later stages of the S phase (Taylor, 1960) and in human cells it was soon shown that there is a strictly ordered sequence of initiation and termination of replication, so that patterns of late labelling could be utilised in identification of some individual chromosomes (Schmid, 1963; German, 1964a). Of particular interest was the latest replicating chromosome of all in female cells, which was the inactive, facultatively heterochromatic X chromosome. As an alternative to auto-radiography, several authors suggested variations in fixation and staining techniques for detection of the 'silent' or inert X chromosome (Saksela and Moorhead, 1962) but these were unreliable.

Several chemicals were known to have fairly specific effects on the appearance of chromosomes (reviewed by Kihlman, 1967) and in 1961, while studying the effects of incorporation

into the DNA of the thymidine analogue 5-Bromodeoxyuridine (BrdU), Hsu and Somers noticed that the secondary constrictions of mouse or Chinese hamster chromosomes were greatly elongated after BrdU treatment (Hsu and Somers, 1961). Later, Palmer used BrdU treatment during the last few hours of culture to accentuate secondary constrictions in an attempt at more detailed classification of human chromosomes (Palmer and Funderbark, 1965). Certain features of individual chromosomes were more consistently enhanced, especially on chromosome pairs 1,9,16 and some B- and D-group chromosomes, and the patterns corresponded in general with late replicating regions seen by autoradiography.

ii. Development of Chromosome Banding Techniques

Unfortunately neither tritium nor BrdU incorporation studies were very satisfactory, mainly because of the problems of timing, especially in human blood cultures where the cells are asynchronous, and to analyse the effects it was necessary to select cells that were treated at the right stage. Moreover, late replication did not distinguish between constitutive and facultative heterochromatin.. A tremendous resurgence of interest in chromosome structure and function therefore followed the recent discoveries of simple staining techniques that allow confident identification of each pair of chromosomes.

a. C-banding

Molecular biologists were interested in the properties of an unusual class of DNA identified as a subsidiary or

'satellite' peak of a different buoyant density from the bulk of mouse DNA, on density gradient centrifugation on Caesium Chloride gradients. This material was christened 'satellite DNA' and was shown to have a characteristic highly repetitious arrangement of base sequences. The development of some of the new staining techniques followed what was really a chance observation in the course of experiments designed to locate mouse satellite DNA on the chromosomes by in situ nucleic acid hybridisation (Pardue and Gall, 1970). The mouse chromosomes were exposed to alkali to denature the DNA, prior to hybridisation with isotope-labelled complementary RNA, and filming for autoradiography. When the autoradiographs were stained with Giemsa it was found not only that satellite DNA was confined to the heterochromatic regions, but also that these centromeric regions and the X chromosome stained intensely with Giemsa, in contrast to the paler chromosome arms. Arrighi and Hsu (1971) developed this technique using alkali treatments prior to Giemsa staining to give intense staining of human heterochromatin, showing up once again the secondary constrictions of chromosomes 1, 9 and 16, and most of the long arm of the Y chromosome, as well as minor sites. Simplified methods were later described (Sumner et al., 1971; Sumner, 1972) and the pattern, described as C-banding (Paris Conference, 1971) at last gave cytologists a reliable method for detection of constitutive heterochromatin, although the facultatitively heterochromatic X was not distinguished by this method. The C-bands also correlated well with the sites of localisation of some classes of repetitious DNA,

and led to new interpretations of the evolution of the karyotype. Experiments with the sort of procedure involved in obtaining these preparations also led to the development of the more comprehensive and now indispensible techniques of G-banding.

b. Q-banding

Following the experiments with plant and Chinese hamster chromosomes stained with fluorescent alkylating agents (Caspersson et al., 1968; 1969), a much more complex pattern of bands along human chromosomes was described by Caspersson and his colleagues in 1970 and 1971. They stained human chromosomes with the fluorochrome Quinacrine Mustard and examined the intricate variegation of fluorescence intensity along the chromosomes. Identical Q-bands were also produced by Quinacrine dihydrochloride (Evans et al., 1971).

c. G-banding

This was soon followed by the discovery of G-banding, where very similar patterns to those obtained with Q-banding were seen in preparations stained with Giemsa after a pre-treatment such as incubation in hot saline (Sumner et al., 1971) or a brief trypsin digestion (Seabright, 1971), and a variety of related G-banding techniques have since been described (Drets and Shaw, 1971; Patil et al., 1971; Schnedl, 1971). Although the G-banded preparations show more precise definition and are permanent, unlike Quinacrine stained preparations, fluorescence analysis is still important in many situations since it shows clearly not only

the intensely stained Y chromosome, inconspicuous in G-banded cells, but also several regions of the karyotype which are polymorphic in size and fluorescent intensity (Evans et al., 1971).

d. Other Banding Techniques

A wide variety of regimens for pretreatment and staining of mitotic chromosomes is now available and besides C-, Q- and G-bands we can produce R-bands, where the Q and G pattern is seen almost completely in reverse, using Acridine Orange (Bobrow et al., 1972b) or Giemsa (Dutrillaux and Lejeune, 1971; Sehested, 1974); T-bands, where telomeres are emphasised (Dutrillaux, 1973); and N-bands, thought to be selective staining of nucleolar organiser regions (Matsui and Sasaki, 1973), or of a particular specialised type of heterochromatin (Faust and Vogel, 1974). N-bands may also be stained using ammoniacal silver (Howell et al., 1975) and this technique has been modified by Goodpasture and Bloom (1975) who found that the material that is stained is non-histone protein. Eiberg has published a technique for staining chromosome satellite material only (1974), and the Giemsa-11 method (Bobrow et al., 1972a) and Giemsa-9 technique (Gagné and Labergé, 1972) stain a certain sub-group of the C-bands, and in particular the secondary constriction of chromosome 9.

e. Mechanism of Banding

Although it is still not entirely clear what the various bands represent in terms of chromosome structure

and composition (see Part I, Chapter Three), investigations of their chemistry as well as their differential susceptibility to breakage, either spontaneous or induced by physical and chemical mutagens, are a rich source of information about chromosome structure and function.

1. Denaturation-renaturation. There are as yet no completely satisfactory explanations of the mechanisms of the specialised chromosome staining reactions. An early explanation for the appearance of C-bands was that the conditions of the treatments cause denaturation of the DNA and that highly repetitive DNA in the heterochromatin is able to renature preferentially. This argument was supported by experiments with the fluorescence of Acridine Orange (AO), but later experiments such as those by Hilwig and Gropp (1972) and Comings et al (1973) showed that returation was not the determining factor in the subsequent reaction with Giemsa, and although Giemsa does bind to DNA (Sumner and Evans, 1973) rather than to protein, it seems that it is an interaction between the DNA and protein components that dictates the final Giemsa intensity. Although there is some evidence for moderately repetitive DNA in G-bands (Sanchez and Yunis, 1974), the preferential denaturation-renaturation argument is not adequate to explain G-banding, as this pattern can be produced in many ways, and trypsin, which should not affect the DNA, produces excellent G-bands. Also, Sumner has shown not only that the hot saline treatment in the ASG technique does not denature DNA, but that Giemsa seems to bind equally well to renatured or denatured DNA.

2. Extraction of Chromatin. Loss of material during alkali and warm saline treatments is involved in the production of C-banded preparations where euchromatin is preferentially denuded (Comings, 1972; Pathak and Arrighi, 1973), and solubilisation and extraction of certain proteins during fixation procedures may be involved in the production of G-bands, but there is controversy over exactly which components may be removed, as claims that histones were removed by acid fixation (Sumner et al., 1973; Comings et al., 1973) were opposed by Pothier et al (1975), who found evidence from immuno-fluorescence that all classes of histones remained in fixed chromosomes. This discrepancy is probably due to the preferential removal of particular types of histones, for instance of H1 (Sumner and Evans, 1973). Any explanation proffered for G-banding must take into account the facts that a) bands can be seen by phase contrast microscopy in fixed, unstained chromosomes, or by electron microscopy of chromosomes without any pretreatment (Bahr and Larsen, 1974); b) that concentrations of protein and DNA do not vary greatly along the chromosomes (Sumner et al., 1973); and c) that fluorochromes such as Quinacrine show up bands without pretreatment, so that the same 'family' of bands is the common end-result of a variety of procedures.

3. Base Composition. Treatment during growth with chemicals such as BrdU or Actinomycin D can induce exaggerated bands, and since BrdU should be substituted for thymine, and Actinomycin D is thought to act on G-C rich regions, their effects suggested clustering of large numbers

of these bases in certain regions of the chromosomes. Caspersson's early argument (Caspersson et al., 1968) that Quinacrine Mustard produced bands because the mustard group bound preferentially to G residues, and was therefore detecting G-C rich regions, was countered by the observation of bands using Quinacrine Dihydrochloride, a compound lacking the mustard group. Quinacrine Dihydrochloride was shown to have enhanced fluorescence when bound in solution to synthetic poly dA, dT (Weisblum and de Haseth, 1972). It seemed (Pachman and Rigler, 1972) that this depended not on the overall concentration of AT bases but on their arrangement in sequences containing four or more adjacent AT base pairs. However, the situation when staining chromatin in situ is obviously different, and this is illustrated by the dull Quinacrine fluorescence of the mouse centromeric heterochromatin that is known to contain AT rich satellite DNA with long 'runs' of AT pairs, although quenching by strategically placed G-C pairs has been offered as an explanation for this (Weisblum, 1973). These regions do have intense fluorescence at interphase, implying that protein interactions in condensation are important in fluorescence, and C-band repetitious DNA is known to be more strongly bound to proteins (Maio and Schildkraut, 1969), while experiments with fluorescent antibodies to adenine (A) where AT-rich C-band regions showed dull fluorescence (Dev et al., 1972; Schreck et al., 1973) confirm that accessibility of DNA for interaction is involved in 'stainability'.

Experiments on the fly Samoaia Leonensis (Ellison and Barr, 1972), kangaroo rat (Bostock and Christie, 1974) and crab Cancer Pugarus (Sumner et al., 1975) among others, have shown that the extrapolation of fluorescent reactions in solution to staining of chromosome preparations is not valid and we cannot necessarily attribute bright Quinacrine fluorescence to the presence of AT-rich DNA in chromatin. Conversely, Daunomycin, although it produces good fluorescent bands, is not markedly affected by base composition of DNA in solution (Comings and Drets, 1976).

The best explanation of the involvement of base composition is that the very intense Q-bands, such as the long arm of the Y chromosome, do have a high AT content and consequent bright fluorescence, while the intensity of the remaining numerous Q-bands is dictated by other interactions also, probably primarily conformation of the chromatin.

4. Conformation of Chromatin. The best explanations so far offered for banding describe the dependence of dye interactions on the folding of the DNA molecule, and since each dye molecule must bind to more than one site on the DNA the spacing of these sites is crucial. The compactness of late-replicating heterochromatic regions allows their intense staining, and the reasons that different sets of conditions may produce on the one hand G- and on the other R-banding may involve the association with interband and band regions of different proteins having characteristic variations in their susceptibilities to denaturation by pH, salt concentration or temperature. This is the mechanism

described by Sumner (1974) who showed, by experiments with chemicals that affect Sulphur-containing proteins in a known way, that there is a relatively higher concentration of protein disulphide groups in positive G-bands, and that these can maintain the compact state of the chromatin and allow intense Giemsa staining. Similarly there seem to be relatively more sulphydryl protein groups in pale G-bands, and if these SH groups are caused to crosslink, thus inducing tighter packing, the pale Giemsa bands are lost. These results correlate with the observation that metaphase chromatin has associated proteins that are disulphide-rich and are not found in interphase (Sadgopal and Bonner, 1970). The compacted state maintained by these proteins may therefore be correlated with genetic inactivity, and histone HI was later shown to be present in condensed but not 'active' chromatin (Simpson, 1974; Gottesfeld et al., 1974). This particular histone is removed by acid fixation of chromatin (Comings et al., 1973) and its loss may be necessary for G-banding as addition of HI protein to preparations of fixed chromosomes blocks Giemsa staining (Brown et al., 1975), presumably by interfering with dye binding. Related ideas on accessibility of dye-binding sites and their possible covering by proteins are discussed by Comings and Avelino (1975).

In conclusion, the most widely supported theories for band induction describe differential packing and conformation of chromatin along the chromosomes, although the same aspect of these variations is not utilised in every staining system

since the experiments that interfered with disulphide or sulphydryl-containing proteins (Sumner, 1974) and abolished G-banding had no effect on the Q-banding patterns.

## B. INCORPORATION OF BrdU

### i. Late DNA Replication

Although the use of BrdU to enhance secondary constrictions as an aid to chromosome identification has been superceded by banding techniques, the use of this compound has formed the basis of a whole new range of investigations in cytogenetics.

In the course of her work on 'late labelling' of human chromosomes with BrdU, Palmer (1970) described the elongated morphology of one of the X chromosomes in a small proportion (about 15%) of female cells, and showed that this appeared only in cells from individuals with XX, XXX and XXY karyotypes or with an abnormal X chromosome, implying that this was the late-replicating X. It was thought that those chromosome segments that completed their DNA replication last would be the last to condense for mitosis, and incorporation of BrdU seemed to prevent efficient 'spiralisation' (Zakharov and Egolina, 1972). In 1974 Zakharov *et al* published results on BrdU incorporation into human chromosomes, confirming and extending Palmer's work and establishing, by using tritiated base analogues, that the underspiralised regions were indeed late-replicating. By altering the timing of addition of BrdU, these authors produced segmented chromosomes whose more elongated regions corresponded to pale G-bands. Since Bromodeoxycytidine affected the same regions, Zakharov *et al* thought the effect was not due to base composition specificity - i.e. the

elongated regions were not necessarily AT-rich - but although their conclusion is correct (see above), the reasoning is not since in most cell types BrdC is deaminated to BrdU before incorporation (Chan, 1973). By using Acridine Orange (AO) fluorescence instead of Giemsa staining the late labelled X chromosome containing BrdU can be clearly distinguished in a much larger proportion of cells (Dutrillaux et al., 1973; Mikkelsen, 1976).

ii. Differentiation between Sister Chromatids

These BrdU labelling techniques have had wide applications, but none so important as the result of another observation by Zakharov and Egolina when they investigated the effects of allowing cells to incorporate BrdU throughout two successive cell cycles so that, due to semi-conservative replication, one chromatid is unifilarly, and its sister bifilarly, substituted with BrdU, causing it to be underspiralised and appear longer than its sister. The crucial property of such chromosomes, however, is that the sister chromatids differ in their staining reactions. Ikushima and Wolff (1974a) were the first to take advantage of the differentiation seen with Giemsa to examine exchanges between the sister chromatids (SCE). Such exchanges had previously been detectable only by autoradiographic methods applied to chromosomes that had undergone one S phase in tritiated thymidine followed by a second replicative phase in normal 'cold' thymidine (Taylor, 1958). A greatly enhanced staining difference between chromatids was described by Latt in 1973. He used a fluorochrome known as

Hoechst 33258, which had been used as a specific stain for regions such as centromeric heterochromatin in mouse cells (Hilwig and Gropp, 1972). Latt showed that when associated with DNA in solution, this dye fluoresced with an intensity that depended on the base composition of the nucleic acid, so that artificial poly-dAdT greatly enhanced fluorescence, while substitution of BrdU into the DNA had a marked quenching effect.

When Hoechst 33258 was applied to chromosomes that had replicated twice in medium containing BrdU (second Metaphase or M2 cells), there was a dramatic difference in fluorescence between the bifilarly and unifilarly substituted chromatids. This very clear differentiation allowed SCE to be scored with confidence, the only disadvantage being the very rapid fading of fluorescence of Hoechst 33258 which necessitated the use of photographs for analysis. This difficulty was overcome by Perry and Wolff (1974) who further developed the technique to the 'fluorescence plus Giemsa' or FPG method to produce very clear and permanent preparations where the chromatid that was brightly fluorescent with H33258 was also more intensely stained with Giemsa. This FPG technique has many applications, for studying metaphase cells after two (M2 cells) or three (M3) cell cycles <u>in vitro</u>, and has also revealed some new information about chromosomes from the appearance of M1 cells that have undergone only one round of BrdU incorporation.

iii. Asymmetrical C-bands

Soon after the description of the Hoechst 33258 technique (Latt, 1973) for differentiation of chromatids in M2 cells, Lin et al (1974) described an unusual staining reaction in the centric heterochromatin of mouse cells, where there was differentiation between the sister chromatids in these C-band regions of M1 cells, although the rest of the chromatin was evenly stained. This 'lateral asymmetry' was attributed to the thymine bias between the strands of mouse satellite DNA (Flamm et al., 1967) such that there was disproportionate uptake of BrdU into the DNA built upon the A-rich strand, resulting in contrast in staining between the chromatids within the satellite DNA-containing regions. These authors also remarked on the contralateral arrangement of the asymmetrical C-bands in metacentric chromosomes that had arisen in the mouse cell line by centric fusion of the acrocentric chromosomes. This was a clear demonstration of the continuity of the DNA of one directional sense through the centromeres, with no change in polarity. The same group (Latt et al., 1974) then reported that there was also discernible asymmetry in the C-band region of the human Y chromosome of M1 cells stained with H 33258, although here the contrast in fluorescence between chromatids was much less marked than in the mouse cells.

The incorporation of BrdU could also be used, then, to find out more about the arrangement of particular sorts of DNA in the chromosomes, and the various staining patterns

in M1, M2 and M3 cells provide a powerful method to be exploited in studies of several aspects of chromosome structure.

## C. APPLICATIONS OF THE NEW SPECIALISED STAINING TECHNIQUES AND AIMS OF THE PRESENT WORK

The new techniques for identifying individual chromosomes and for highlighting specific regions of the chromosome complement have been widely exploited in the last few years, and led to many advances in cytogenetics including an extension of our knowledge of chromosome polymorphisms and rearrangements. Variations in sizes of secondary constrictions had been observed using conventional staining techniques such as orcein staining (e.g. Lubs and Ruddle, 1971) and the use of C-banding techniques has revealed a wide range of sizes of the intensely staining regions of human chromosomes, and in particular of chromosomes 1, 9, 16 and Y, in phenotypically normal individuals. Polymorphisms of a different sort have also been revealed by the use of Quinacrine fluorescence, where regions such as the satellites of the acrocentric chromosomes and several bands have been shown to vary in size and in fluorescent intensity (e.g. Evans et al., 1971). The very intense Quinacrine fluorescence of the distal part of the long arm of the Y chromosome has been useful in studies of rearrangements, and also led to the development of a simple test for male sex chromatin, visible as brightly fluorescent bodies ('Y bodies') in interphase cells, and forming a useful test, complementary to the use of 'Barr body' studies for female sex chromatin, in buccal smear examinations. The G- and R-banding techniques have also been widely used, and the introduction of banding methods led to the accurate

identification of the chromosomes involved in rearrangements in constitutional anomalies (reviewed by Dutrillaux and Lejeune, 1975), and the extra chromosome in conditions such as Down's syndrome (trisomy 21) and trisomy C (very often trisomy 8 - e.g. de Grouchy et al., 1971).

These are but a few examples of the tremendous possibilities opened up by the use of new staining techniques, but one problem that had not until now been studied using 'banding' was the interesting case of the reported increase in aneuploidy in blood lymphocytes associated with increasing age and first described by Jacobs and her colleagues in Edinburgh in 1961. Many further studies all over the world have extended and confirmed the observations that aneuploidy increased with age and that the pattern of chromosome loss and gain showed a sex difference, especially evident in the disproportionate number of female cells that had lost or gained a medium-sized submetacentric, or C-group, chromosome (Jacobs et al., 1963). (These studies are described in detail in the introduction to part II of this work). There was some evidence for preferential loss of the Y chromosome from male cells, so that Jacobs et al (1963) postulated that the C-group chromosome lost so commonly from female cells might well be an X chromosome, and in the present study I set out to analyse cells from a random sample of the population to identify the chromosomes involved in aneuploidy, using G-banding, and to determine, should the X chromosome indeed be lost more frequently, whether this loss was random or involved preferentially the

inactive, or active, X chromosome.

With this in mind, I was experimenting with incorporation of 5-Bromodeoxyuridine (BrdU) in the later stages of the S phase as a method of identification of the late-replicating X chromosome (see above), when the method for differentiation between sister chromatids after two cycles of BrdU incorporation was published (Latt, 1973) and the FPG method for obtaining permanent preparations of 'Harlequin' chromosomes was developed in this laboratory by Perry and Wolff (1974), using a Chinese hamster (CHO) cell line. I went on to apply this technique to human lymphocyte chromosomes to investigate several problems that had previously been studied by the autoradiographic (ARG) method (see part I, chapter one) and had not been satisfactorily solved due to the lack of resolution of ARG techniques; and also to study such properties as the distribution of sister chromatid exchanges (SCEs) throughout the human chromosome complement, and their frequency in individuals of all ages, with constitutional chromosome abnormalities, or suffering from the 'chromosome breakage syndromes' Ataxia Telangtiectasia and Fanconi's Anaemia. The central problem of interest in these studies was the relationship of SCE to gross chromosomal abberations and to cellular repair methods for damage to DNA, so that studies on lymphocytes exposed to mutagens were also undertaken using normal and repair-deficient cells (see Part I, Chapter Five).

# PART I

## INVESTIGATIONS OF CHROMOSOME FUNCTION AND STRUCTURE USING INCORPORATION OF 5-BROMODEOXYURIDINE

# CHAPTER ONE

## INTRODUCTION

## A. INFORMATION ON CHROMOSOME STRUCTURE FROM AUTORADIOGRAPHY

The powerful new staining techniques for detection of chromatin that has incorporated the base analogue BrdU (Latt, 1973; Perry and Wolff, 1974) have now made it possible to investigate thoroughly some of the problems of chromosome structure and function that have occupied many workers for more than a decade, since the limited resolution of autoradiography (ARG) resulted in conflicting data. The difficulties in interpretation of data from ARGs left unsolved the problems of whether exchanges between sister chromatids (SCE s) occur naturally or only when induced by radiation, and whether chromosomes are composed of a single continuous DNA double helix (unineme) or two or more DNA molecules (bi- or polyneme), and what arrangement or directional sense these molecules may adopt.

### i. Spontanaeity of SCE

One basic uncertainty still remains. It is not finally established whether SCEs occur spontaneously or are all induced by the procedures necessary to detect them. Early experiments with tritium did not reveal a marked effect of tritium dose on SCE frequency (Marin and Prescott, 1964) and were thought to show that SCEs were not induced

by the endogenous radiation from tritium. However, Wolff (1964) argued that the lack of a tritium concentration effect was due to a saturation phenomenon, so that little increase in SCE frequency could occur above a certain level already induced by the dose of isotope necessary to detect SCE. This argument was supported by the increase in SCE caused by tritium in X-irradiated endoreduplicated cells (Olivieri and Brewen, 1966) and by the saturation effect seen after exposure to X-rays, when the SCE frequency in tritium-labelled cells increased with dose of radiation only up to a certain maximum level, after an X-ray dose of 50r (Marin and Prescott, 1964). The tritium dose effect and plateau level was demonstrated in 1972 by Gibson and Prescott, confirming that at least some of the SCEs were radiation induced.

There was evidence for the natural occurrence of sister strand exchanges in ring chromosomes in several systems (McClintock 1938; Brewen and Peacock, 1969a) since SCE between ring chromatids can lead to the formation of a dicentric ring. It seemed therefore that there was probably a low spontaneous level of SCE but that ionising radiation, either in the form of endogenous isotope or exogenous X-rays (Marin and Prescott, 1964), or UV light (Rommelaere et al., 1973), could increase the numbers. The saturation effect was a mystery since various auto-radiography studies in plants (Taylor, 1958; Peacock, 1963) and animal cells (Taylor, 1965; Gibson and Prescott, 1974) had shown that the SCEs formed seemed to be randomly

distributed according to the length of the chromosomes, so that there did not seem to be any restriction on the sites available for exchange.

ii. Isolabelling and Subunit Rejoining

In ARG_s of cells in their second mitosis (M2) following a pulse treatment with $^{3}$H-thymidine in the first cell cycle, certain chromosome regions were seen where there was a similar amount of label over corresponding sites on sister chromatids instead of the usual pattern with an unlabelled segment immediately opposite a labelled one. This situation, described as 'isolabelling', was used as evidence for a multineme model of chromosome structure (Peacock, 1963), or for an exhange of only one strand (half-helix) between the DNA double helices in each chromatid (single polynucleotide strand exchanges; Gatti and Olivieri, 1973; Gatti et al., 1974; fig. I, 4, 5). A strong piece of evidence that the chromosome is mononeme came from Taylor's autoradiographic studies (1958) of SCE in tetraploid cells. Such cells contain duplicate SCEs affecting both sister chromosomes at the same site ('twins' of SCEs that occurred in the first cycle after labelling), as well as 'single' SCEs that are represented only once, and occurred in the second post-label cycle, immediately before observation. The ratio of singles to twins is dictated by the way in which chromosome subunits can rejoin when forming an exchange (see fig. I, 4, 3). Taylor's results, where the ratio of singles to twins was 2:1, were equivalent to those predicted on the basis of non-random rejoining of

the subunits - i.e. the rejoining is restricted, presumably by the polarity of the DNA molecules. Further evidence for non-random rejoining came from observations on tritium-labelled dicentrics, where rejoining was much more likely to occur between 'like' than 'unlike' chromatids as defined by their labelling patterns in ARG s (Brewen and Peacock, 1969b). However, conflicting results in Haplopappus cells where Sparvoli and Gay (1973) found a single to twin ratio of 10:1, a ratio expected if rejoining were random, left this problem unsolved.

The interpretation of autoradiographic studies of SCE had thus not provided unequivocal evidence for one or other of the polyneme or unineme models of chromosome structure and also left in doubt the issue of the nature of SCE events.

## B. AIMS OF THE PRESENT WORK

In this study the new FPG technique has been applied to human chromosomes to investigate their staining properties, and to try to solve some of the problems posed by conflicting ARG information. While the emphasis in this work is on human chromosomes, limited studies on mouse and sheep cells were also undertaken. Since SCEs were thought to be an indication of repair and/or mutation, it was of interest to investigate the incidence of SCE in groups of individuals of different ages, and in patients with abnormal chromosome constitutions such as translocations or trisomy 21, or with a history of X-ray exposure, such as in the treatment of ankylosing spondylitis. Lymphocytes from patients with chromosome breakage syndromes were also examined since these cells have defective DNA repair systems, and their response to various mutagen treatments *in vitro* was investigated for comparison with normal cells. This kind of study was designed to examine the relationship between SCE, abnormalities of repair and the occurrence of chromosome aberrations.

# CHAPTER TWO

## A. MATERIALS AND METHODS

### i. Blood Culture

a. Human Blood. Blood samples were taken from adults by venupuncture, and obtained from newborn babies by heel-prick or from cord blood. The culture method was essentially that of Hungerford (1965). Whole blood (0.8 ml adult venous blood, 0.4 ml cord blood, or 0.1 ml heelprick sample) was added to 9 ml medium (Ham's F10, RPMI 1640, McCoy's or Eagles MEM) containing:

10-15% newborn or foetal calf serum
0.01% Phytohaemagglutinin (PHA, Wellcome)
100 units per ml Penicillin
0.1 mg/ml Streptomycin (Glaxo)
Glutamine (0.03%) was also added to all media except F10.

After an incubation time of two or three days at 37° C, Colcemide was added to a final concentration of 20 μg/ml ($2.7 \times 10^{-5}$M) for the last 2.5 to 3.5 hours of culture. Suspensions were treated with a hypotonic solution of 0.075M KCl for a total of 15 minutes, and washed three times in 3:1 Methanol:acetic acid fixative. The suspension was dropped onto glass slides to make standard air dried preparations.

b. Sheep Blood. The culture method was the same as that used for human blood, but the medium had the following composition:

Ham's F10
10% foetal calf serum
10% tryptose phosphate broth
0.08 ml (4 drops) purified PHA per 10 ml culture
0.04 ml (2 drops) heparin per 10 ml culture (0.4%)

ii. Human Fibroblast Cultures

These were grown on plastic Falcon flasks or in large Leighton tubes containing washed glass microscope slides. The medium used was Ham's F10 containing antibiotics, 0.03% Glutamine, 20 mM Hepes buffer at pH 7.2, and 5 to 20% foetal calf serum. Cells were removed for subculturing after washing with Dulbecco's phosphate buffered saline (PBS), by treatment at 37° C with a mixture of 0.1% trypsin and 0.02% versene for about five minutes, after which cells were collected in medium containing serum to stop the action of trypsin. Trypsinised cells from cultures that had been treated for about three hours with $2 \times 10^{-7}$ M Colcemide could be used for chromosome preparations, and hypotonic and fixation treatments were the same as those used for blood cultures. When cells were grown on glass slides, the whole slide was processed by washing with PBS, and treating with KCl for up to 40 minutes, followed by a 1:1 mixture of KCl and fixative for 10 minutes, before fixation in 3:1 fixative.

iii. A9 Mouse Cell Line

These cells were grown in McCoy's medium, or in RPMI 1640 containing 15% foetal calf serum, Glutamine, antibiotics and pH 7.2 Hepes buffer. As an alternative to fixation in suspension, cells could be fixed on the slides after hypotonic treatment, by a method suggested by Dr. C.J. Bostock. The slides were flooded with fixative and the excess liquid drained off before adding a drop of cell suspension. Cells could now be deposited on the slide by careful addition of small drops of fixative on top of the drop of cells, and round the edge of the drop. This often gave more satisfactory spreading of chromosomes.

iv Staining

a. G-banding. The ASG method of G-banding (Sumner et al., 1971) was found to give more reliable results than trypsin banding. Slides were usually allowed to age for one to seven days before incubation in 2 x SSC[1] at 60° C for two to 18 hours, depending on the age of the slide, and staining in 5% Giemsa in Gurr's phosphate buffer, at pH 6.8, for three to 10 minutes. Preparations were then dried and mounted in DePex.

b. Q-banding. This was carried out by the method of Evans et al (1971), using 0.5% Quinacrine Dihydrochloride in distilled water for six to 10 minutes, followed by gentle washing in running water for about three minutes, and mounting in deionised water.

[1] 0.3 M sodium chloride plus 0.03 M trisodium citrate

c. C-banding. This was carried out by the method of Sumner (1972). Slides were allowed to age for at least three days before treating with:

0.06 M HCl for one hour to clear the cytoplasm
5% BaOH at 50° C for five to 10 minutes
2 x SSC at 60° C for one hour
5% Giemsa in phosphate buffer (pH 6.8) for five to 15 minutes.

d. R-banding. This was achieved by the heat treatment described by Dutrillaux and Lejeune (1971), or simply by staining untreated slides in 0.005% Acridine Orange freshly dissolved in M/15 Sorensen's buffer at pH 6.7 (Mikkelsen, 1976). Slides were mounted in the same buffer. Giemsa R-banding was obtained by the method of Sehested (1974), incubating slides in sodium orthophosphate (pH 4-4.5) at 88° C for up to 10 minutes, and staining in 5% Giemsa in water instead of buffer.

v. Fluorescence Microscopy

A Leitz Ortholux microscope with a HBO 200 W mercury lamp and Ploem vertical illuminator was used. For preparations stained with Quinacrine or Acridine Orange (AO), Ploem setting number three, excitation filter BG12, and interference filter 510 nm were used, while for viewing Hoechst 33258 stained preparations the Ploem setting was number one, excitation filters were UG1 and 5, with a gray red suppression filter, and the interference filter used was 430 or 460 nm.

vi. Photography

For photographing G-banded or Q- or AO-fluorescence the film used was Kodak Panatomic-X, while for high-contrast preparations such as autoradiographs and C-banded cells Microfile was better. A 'faster' Tri-X film was used for photographing Hoechst 33258 fluorescence.

vii. Autoradiography

G-banded slides were destained by soaking for three days in several changes of 70% alcohol, followed by several hours in 3:1 methanol:glacial acetic acid. Slides were dipped in Ilford L4 emulsion dissolved in distilled water at 50° C, and dried at room temperature before storing in light-tight boxes at 4° C. Preparations were then developed in Kodak D19 for four minutes, rinsed in water, fixed in Kodafix diluted 1:4 with water, for four minutes, and washed for at least 20 minutes before staining through the emulsion with Giemsa. Very small silver grains were obtained by using chilled solutions for processing slides that had just been taken from the refrigerator, and by developing for only two minutes (Peacock, 1970).

viii. X-Irradiation Experiments

Blood was irradiated in glass bottles after mixing with growth medium. A Siemens Stabilipan X-ray machine was used, with a copper (.8 mm), tin (.25 mm), and aluminium (1 mm) filter, giving a dose rate of about 50r per minute when run at 250 KW and 15 mA.

## ix. UV-Irradiation

A Hanovia low pressure mercury vapour lamp (ARC type 12555), giving UV of the 254 nm range, was used for irradiation of fibroblast cultures. Cells were grown on glass microscope slides or coverslips and washed once in PBS at 37° C before placing on sterile petri dishes for irradiation, and returned to culture vessels containing growth medium. To detect unscheduled DNA synthesis (Rasmusen and Painter, 1964) cells were grown to confluency, treated with medium containing only 1% serum for 24 hours to reduce the numbers of cells in S phase, irradiated, and replaced in medium containing 15% serum and high specific activity (10 μCi per ml) tritiated thymidine (Cleaver, 1968). After a three-hour labelling period, slides were washed with PBS, fixed in 70% alcohol followed by 3:1 methanol:acetic acid, dried, and dipped in L4 emulsion. After an exposure time of one week, slides were processed and stained through the emulsion with Giemsa.

## x. Chemical Mutagens

Mitomycin (MMC; Kyowa, Dales Pharmaceuticals) and Adriamycin (AM, Pharmitalia) were obtained as powders and Ethyl Methane Sulphonate (EMS, Sigma) as a liquid. All three were stored at 4° C and were dissolved and diluted with distilled water immediately before use. Precautions were taken to avoid exposure to these mutagens and where possible the solutions were kept in bottles with rubber seals to allow withdrawal of the solutions by syringe.

xi. BrdU Incorporation

Thymidine-free media were used to allow maximum incorporation of BrdU. Eagles MEM, McCoy's and RPMI 1640 were all tested and the most satisfactory medium was RPMI 1640. Cultures were protected from light by wrapping bottles in black plastic.

BrdU (5-Bromodeoxyuridine, Sigma) was dissolved in distilled water and 0.1 ml amounts from stock solutions were added to 10 ml cultures to achieve final concentrations of 5 μM to 1.5 mM BrdU. For some experiments 5-Fluorodeoxyuridine and Uridine were also added to give final concentrations of 0.4 μM FUdR and 6 μM UdR. FUdR was a gift from Roche Products.

a. 'Late Labelling' with BrdU. Concentrations of BrdU ranging from 100 to 400 μg per ml were added to blood cultures during the last three to eight hours of incubation. In slides stained simply with Giemsa, the despiralised X chromosome was most frequently seen after 3.5 hours of BrdU incorporation. These high concentrations of BrdU are necessary for marked alteration of chromosome morphology. For detection of the late-replicating X chromosome using AO fluorescence, lower concentrations of BrdU were used.

b. FPG Technique for Differentiation of Sister Chromatids. Hoechst 33258 was the gift of Dr. H. Loewe of Hoechst Pharmaceuticals. The powder was dissolved in distilled water and a stock solution at 50 μg per ml was stored at 4° C, retaining its efficacy for several months. The

stock was diluted 1 in 100 with water immediately before use.

Slides were stained in Hoechst 33258 at 0.5 μg/ml in deionised water for 12 minutes, rinsed in water, and mounted in water or MacIlvaine's buffer (Latt, 1973) - i.e. 0.16 M sodium phosphate, 0.04 M sodium citrate, pH 7. Coverslips were sealed with rubber solution. At this stage the slides could be examined under fluorescence microscopy, or further treated with light as follows:

1. Light from fluorescent tube of X-ray viewing box for one to six hours;

or 2. Mixture of daylight and fluorescent light on the bench, for two to 30 hours;

or 3. UV light, unfiltered, from the 200 W mercury lamp of the microscope, for 30 to 45 seconds.

After the light treatment the coverslips were removed and slides were rinsed in water and incubated in 2 x SSC at 60° C for 30 minutes to two hours, and stained in 5% Giemsa in phosphate buffer at pH 6.8 for three to 10 minutes.

An alternative stain to Hoechst 33258 is Acridine Orange (AO) used as described above. Here the fluorescence is more stable but the differentiation between chromatids increases under UV microscopy and the subsequent treatments and Giemsa staining will not result in differentiated chromatids without this prior UV exposure.

## B. INVESTIGATION OF THE FPG STAINING TECHNIQUE

### i. Giemsa

After incorporation of BrdU during two cell cycles, a difference between sister chromatids could be detected using simple Giemsa staining, especially after using higher concentrations of BrdU - e.g. above 150 μM - when the bifilarly substituted chromatid was slightly paler and underspiralised compared with its sister, and the chromosomes often looked curved because of this longer chromatid.

### ii. Fluorescence

After staining in Hoechst 33258, cells that had undergone only one round of BrdU incorporation (M1 cells) showed a banding pattern very like Q-banding, with intense fluorexcence that faded very rapidly compared with that of Quinacrine. After two rounds of incorporation, the dramatic differentiation between chromatids was seen (M2 cells; Fig. I, 2, 1a), while after three rounds (M3 cells) only one quarter of the chromatin still had the original unsubstituted DNA and fluoresced brightly, so that many chromosomes now had two dull chromatids (Fig. I, 2, 1b).

The Hoechst 33258 was used dissolved in water (0.5 μg per ml), and the buffers used by Latt (1973) for stain dilution and mounting could be dispensed with. However, if water was used as a mountant for examination by fluorescence, chromosomes were liable to swell up, particularly in very fresh preparations, so that a mounting buffer was used.

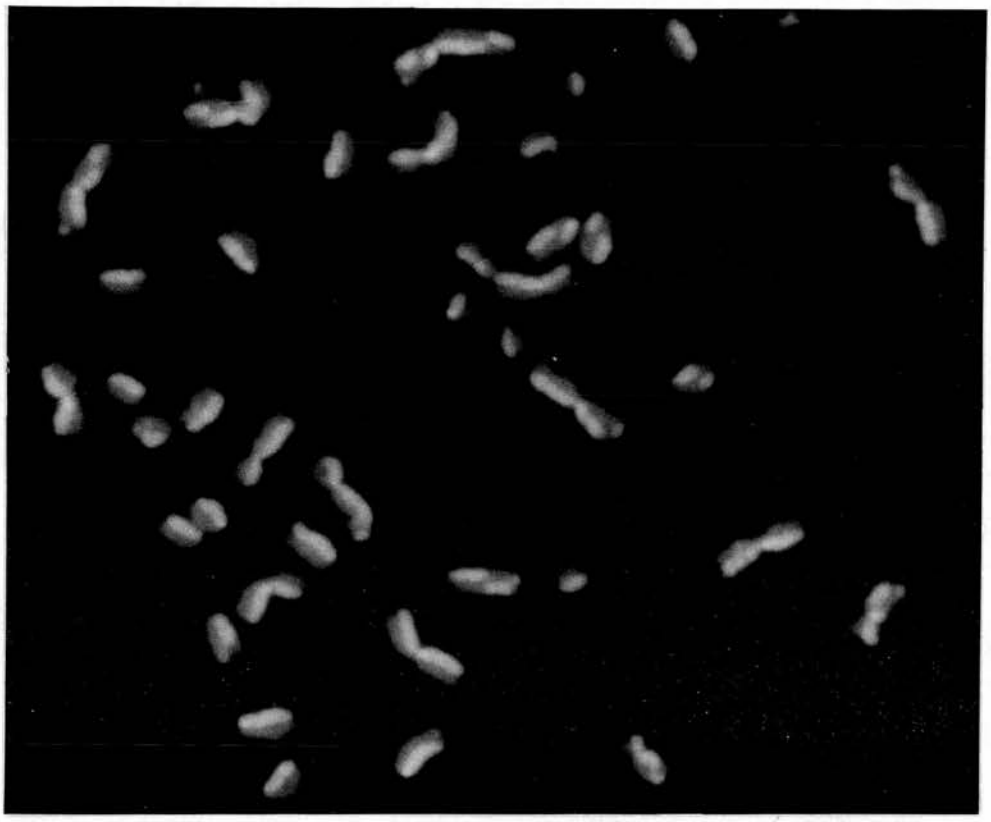


Fig. I, 2, 1a.

Mouse chromosomes (A9 cell line) stained with the fluorochrome Hoechst 33258, after undergoing two cell cycles in the presence of BrdU (M2 cell). There is clear differentiation in fluorescent intensity between sister chromatids; (the dull chromatid is bifilarly, and the bright chromatid unifilarly substituted with BrdU), and sister chromatid exchanges (SCEs) are distinctly seen.

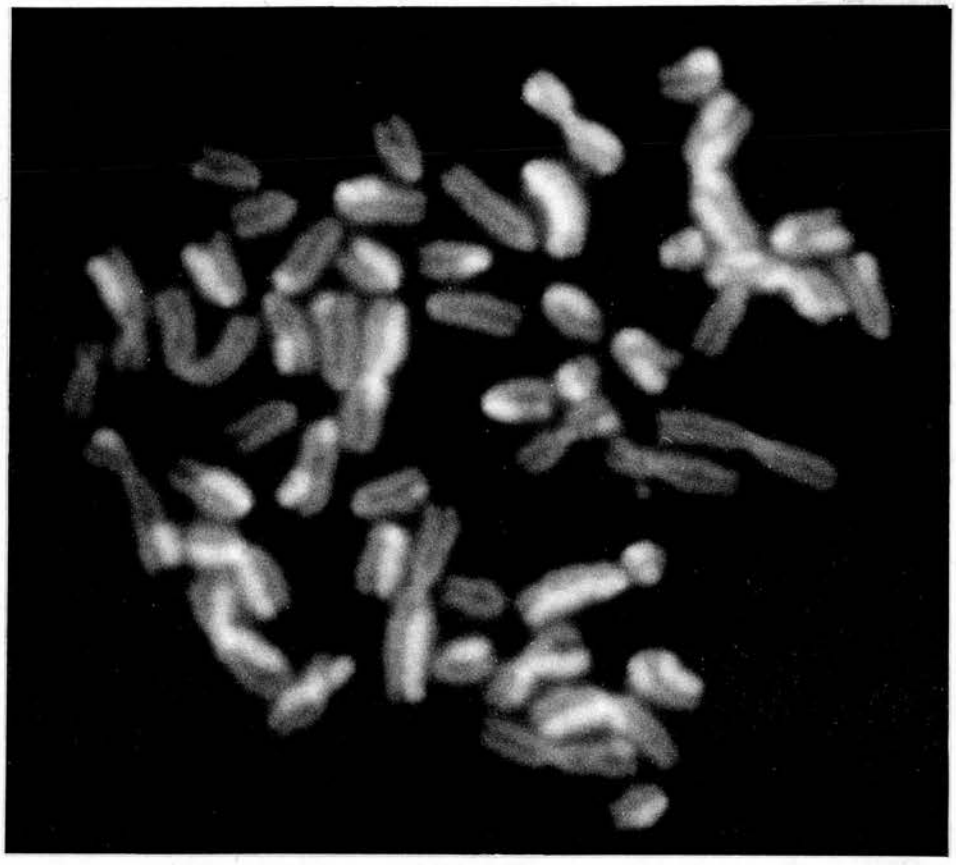


Fig. I, 2, 1b.

Mouse chromosomes stained with Hoechst 33258 after three cell cycles in the presence of BrdU (M3 cell). Only one quarter of the chromatin is now brightly fluorescent since it contains the remaining unifilarly-substituted DNA.

At first, preparations were mounted and examined by fluorescence microscopy before further treatment, but it was found that Hoechst fluorescence faded in a matter of a few seconds, and cells that had been 'bleached out' in this way were subsequently unable to take up Giemsa stain. Thus, although the fluorescence microscope was used as a quick check of quality of preparations, exposure was kept to a minimum if permanent preparations were needed.

iii. Fluorescence (Hoechst 33258) plus Giemsa

Staining in Giemsa with or without treatment for one to 20 hours in 2 x SSC immediately after Hoechst 33258 staining did not give good differentiation between chromatids, and an ageing period in light was required to improve the effect. Various types of light exposure were tested.

a. Mounted slides.

1. Exposure to a mixture of sunlight and fluorescent light on the bench for one to 48 hours.

2. Light from a fluorescent X-ray plate viewing box, either filtered through opaque glass with the slides resting on top, or with the box inverted over the slides, and using aluminium foil as a reflector on the bench.

3. Germicidal UV for 10 minutes to 24 hours at a distance of 9" to 24" from the tube.

Fig. I, 2, 2a

"Harlequin chromosomes". Human lymphocyte chromosomes (at M2) stained by the Fluorescence-plus-Giemsa (FPG) technique. The chromatids that are bifilarly substituted with BrdU and hence dully fluorescent when stained with Hoechst 33258, are also pale-staining in these harlequin chromosomes.

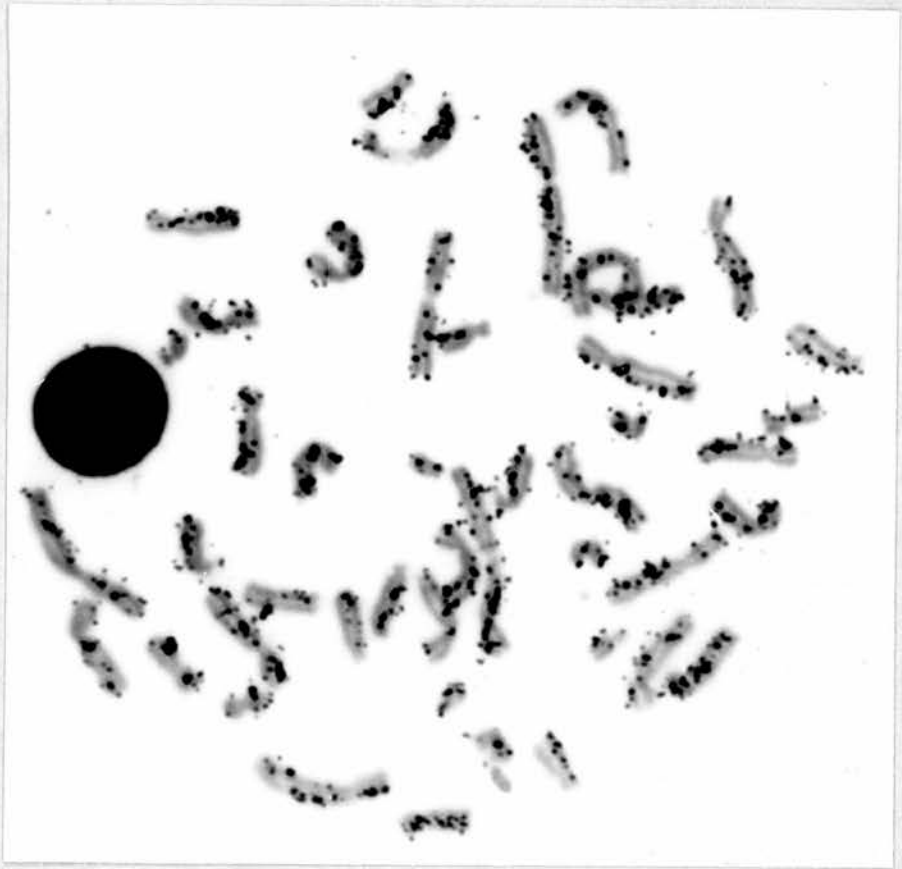


Fig. I, 2, 2b.

Autoradiograph of human chromosomes that have undergone one cell cycle in the presence of tritiated thymidine, followed by a second round of DNA synthesis in normal medium containing "cold" thymidine. This illustrates the lack of resolution of ARG in studies of SCEs, when compared with the FPG-stained "Harlequin-stained" chromosomes shown in Fig. I, 2, 2a.

4. Incident light from the UV microscope with the objective removed - i.e. UV from the 200 W mercury lamp filtered through excitation filters UG1 and 5 and the interference filter 430 nm, for 10 to 60 seconds.

5. Unfiltered light from the 200 W mercury lamp at a distance of about 1" from the bulb, for 10 to 60 seconds.

6. UV light from a Hanovia low pressure mercury vapour lamp for 10 to 120 seconds.

b. Unmounted slides.

Methods 1, 2, 3 and 6 were also tested on unmounted slides covered with a layer of buffer or sealed in a glass moisture chamber containing blotting paper soaked in water or buffer. Following the light treatment, coverslips were removed and slides rinsed with water and treated with 2 x SSC at 60° C for 10 minutes to 20 hours, before rinsing in water and staining for three to 15 minutes in 5% Giemsa.

The conclusions from all these experiments were that the most reliable and consistent technique for obtaining 'harlequin chromosomes' (Wolff and Perry, 1974) on human lymphocyte preparations was that described on page 35 (Fig. I, 2,2). Apparently UV light is not essential as UV is not transmitted through glass, and longer wavelengths must be involved. Light exposures other than sunlight or fluorescent

light from the X-ray viewing box were effective, but the UV microscope was inconvenient, the germicidal UV required lengthy exposure, and the UV mutagenesis lamp could be used for only one or two slides at a time, like the microscope. The age of the preparation before staining had quite a marked effect on the sort of treatment needed for the best results, and I also found that the longer the light exposure, the paler the final preparations. This bleaching effect also appeared after longer incubation times in 2 x SSC. It was clear from sequential staining of the same cells that the dully fluorescent chromatid is also the one that stains less intensely with Giemsa, and incorporation of tritiated BrdU was used to show that this is also the doubly substituted material.

iv. Acridine Orange

Unlike H 33258-stained preparations, AO stained cells required exposure to UV before differentiation between chromatids develops. There was a relatively slow improvement in contrast up to a maximum during 30 to 60 seconds' viewing, when one chromatid was bright yellow-green and the other was dull orange-red, after which fading took place. This fairly intense exposure to UV was necessary for successful differentiation with Giemsa, and the best preparations were obtained from cells that were exposed just long enough to give maximum contrast of fluorescence. Overlong exposure to UV again resulted in chromosomes that could not take up Giemsa. Since the AO solution used had a concentration of 0.005% and was therefore 100 times stronger than the H 33258, I

tested diluted AO to see if this would preclude the UV treatment, but the fluorescent result was poor, with dull yellowish-brown fluorescence throughout. In some cells there was chromatid differentiation, and this did develop faster than with more concentrated AO, but the subsequent 2 x SSC and Giemsa treatment did not give such good 'harlequins' as the H 33258 or 0.005% AO methods.

v. Quinacrine Dihydrochloride

This dye was also tested, at the routine concentration of 0.5% used for Q-banding, or diluted to 0.005% for comparison with AO. There was some differentiation between chromatids with either concentration of Quinacrine, and the contrast developed and faded faster than in AO stained preparations. Scoreable 'harlequin' preparations were obtained with the FPG technique using Quinacrine, but the results were much less reliable than with the other two fluorochromes.

vi. Other Techniques for Producing Harlequins

a. Hot Alkali

Application of C-banding technique to BrdU substituted cells not stained with H 33258 produced some clear, differentiated cells after the following procedure:

4 minutes BaOH at 50° C

1 hour 2 x SSC at 60° C

5 minutes 5% Giemsa

However, this was not at all reliable, and replacing the BaOH with Sorensen's buffer at pH 9 did not produce harlequins. Some time after this, Korenberg and Freedlender (1974) published a similar hot alkali method for obtaining harlequins without using H 33258; this involved incubation for 10 minutes at 87°- 89° C in a 1M solution of $NaH_2PO_4$ at pH 8, followed by staining in Giemsa. I have found this is less reliable than the FPG technique as it does not always give harlequins and the chromosomes tend to have a 'fuzzy' appearance.

b. Hot Saline

Incubation of H 33258-stained and light-treated preparations in 2 x SSC at 84°-87° C for short periods of about 10 minutes was also effective, although paler preparations resulted from this heat treatment, and longer staining times of up to 30 minutes in 5% Giemsa were required. Miller et al (1976) stated that incubation of such slides in water at 60° C or 84° C produces good harlequins, and that the higher temperature gives improved results on slides where the amount of BrdU incorporation is low. However, I find that this water treatment swells and bleaches the chromosomes badly, and treatment in hot 2 x SSC is preferable.

vii. G-Banding and Harlequins

G-banding was seen in harlequin preparations, especially in fresh slides that are given the least intense treatment such as short light exposure, and the banding may be more

pronounced in the dark chromatid, or may show up as 'ghost' G-bands on the pale chromatid also. In general, G-banding complicates the scoring of SCE, as a dark band appearing on the paler chromatid can mimic a small exchange, and in preparations with G-bands the differentiation between chromatids was usually less clear, and did not allow easy assignment of exchange points to bands.

The best G-banding was seen in new slides (two to four days old) after a fairly short light exposure and one to six hours in 2 x SSC at 60° C. G-banding was also produced by a short heat treatment in hot 1M Sorensen's buffer at pH 6.8 to 7.0 (up to 10 minutes at 88° C) followed by staining in Giemsa (Fig. I, 2, 3).

Trypsin-banding methods were also tested with or without prior H 33258 staining and light exposure. Trypsin was used at a final concentration of 0.001% in 0.025 M Sorensen's buffer (pH 6.8) containing 2% Giemsa. Treatments at room temperature for three to eight minutes produced banding of H 33258-stained cells, but this was most clear in slides that had not been light exposed and therefore were not showing very clear chromatid differentiation. I also tested trypsin at 0.1% dissolved in saline. Fifteen to 60 seconds' treatment at room temperature was followed by rinsing and staining in Giemsa. With slides that had been stained with H 33258 and light treated there was a progression through increased time in trypsin from chromosomes with pale chromatid only banded,

Fig. I, 2, 3.

"Harlequin" chromosomes, also showing G-banding. This preparation was stained with Hoechst 33258, exposed to light , and incubated for 10 minutes in 1M Sorensen's buffer at 88°C (pH 6.8), before staining with Giemsa.

to both chromatids banded, to dark chromatid only banded, until overdigestion resulted in swollen 'empty' chromosomes. The pale chromatid thus seemed to be more sensitive to trypsin digestion, and by the time both chromatids were banded the harlequins had almost disappeared, so that this method was not very satisfactory for producing harlequins and G-bands together.

viii. R-Banding and Harlequins

AO staining of BrdU substituted chromosomes produced R-banding in M1 cells, and these R-bands were also seen in M2 cells until the chromatid differentiation developed during UV exposure while viewing. Cells showing both harlequin staining and R-bands could be obtained after the usual H 33258 and light treatment followed by a heat treatment in 1M $NaH_2PO_4$ at pH 4.2 and staining in Giemsa in water (Sehested, 1974), but once again it was found that good banding and clear harlequins rarely co-exist. It was possible to carry out a series of staining procedures on the same slides, such as photographing Quinacrine-stained cells, washing out the dye, and following the usual FPG procedure; but if R-banding or ASG methods were used and the Giemsa removed with alcohol, the FPG technique could no longer give harlequins on these cells.

It is interesting that after the hot treatment at low pH required for R-banding, if slides were not stained immediately with Giemsa, but were treated instead by the FPG method, harlequins were still produced but the R-bands were abolished.

# CHAPTER THREE

## SCOPE OF THE FPG TECHNIQUE

### A. LATE-LABELLING WITH BrdU

#### i. Giemsa

Concentrations of BrdU ranging from 100 to 400 μg per ml (or 0.325 mM to 1.3 mM) were added to the blood cultures for the final three to eight hours of incubation. Using simple Giemsa staining, a despiralised chromosome, the putative late-replicating X chromosome, was visible in a proportion of cells (Table I, 3, 1) and this was most frequently seen after 3.5 hours of BrdU incorporation, while with increasing time of BrdU exposure fewer differentiated X chromosomes were seen and there was more emphasis on the secondary constrictions of chromosomes 1, 9 and a D group chromosome, probably 13, as well as a B group member, probably chromosome 4. BrdU also caused some chromatid aberrations, and after treatment at 200 μg per ml of the analogue, up to 15% of the cells had chromatid gaps. The late-labelled cultures from males also show an indistinct staining pattern on the Y chromosome, where the C-band region often stained pale and looked 'fluffy'.

#### ii. Acridine Orange

The use of Acridine Orange (AO) fluorescence allowed the late X to be detected in many more

TABLE I 3 1

Late Labelling with BrdU; Identification of Late-replicating X chromosome using Giemsa stain only.

| BrdU added; (hrs) | Conc. BrdU (mM) | Cells with chromatid gaps | Cells where X identified | Total cells |
|---|---|---|---|---|
| 5 | 0.325 | 3 | 2 | 20 |
| 4.5 | 0.325 | 0 | 8 | 20 |
| 4 | 0.325 | 4 | 4 | 20 |
| 3.5 | 1.3 | 2 | 6 | 20 |
| 3.5 | 0.65 | 5 | 8 | 30 |

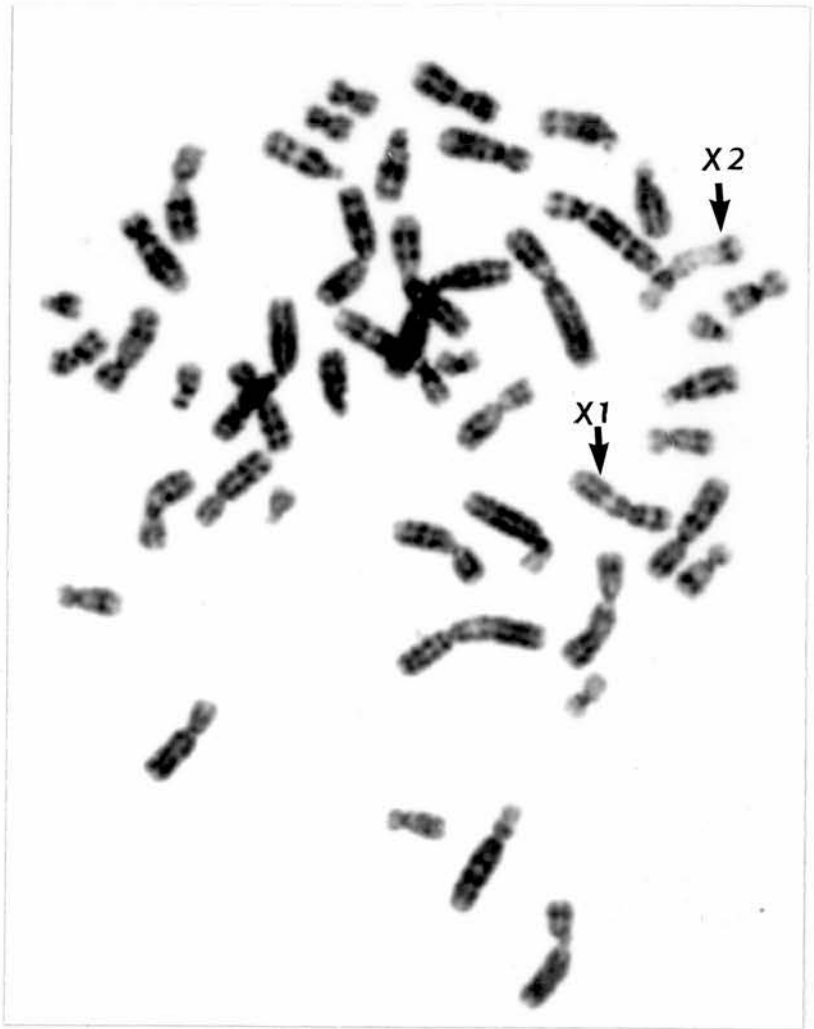



Fig. I, 3, 1.

Female chromosomes that have been "late-labelled" by treatment with BrdU during the final 5 hours of culture, and stained by the FPG technique. The regions of the complement that contain BrdU, i.e. were undergoing DNA synthesis towards the end of the S phase, are pale staining. The late-replicating X chromosome (X2, arrow) is conspicuous due to its pale stained appearance, and the "early X" (X1, arrow), shows R-banding.

cells, since the fluorescence intensity and colour were affected at incorporation levels where the condensation of the chromosome was not markedly affected.

iii. Fluorescence plus Giemsa

Areas of the karyotype that contain more BrdU can be expected to show dull H 33258 fluorescence and pale staining with Giemsa. Addition of the BrdU during the later parts of the S phase resulted in chromosomes showing an R-banded pattern, confirming that the dark G-bands seen by ASG staining are later-replicating areas (Ganner and Evans, 1971). The asynchrony of growth of blood lymphocytes leads to a wide variety of staining patterns because cells take up the analogue during varying proportions of the S phase.

With the FPG technique, using the same methods described above for obtaining harlequin chromosomes, the results were repeatable and allowed positive identification of the 'late' X chromosome in 65-75% of the cells (Fig. I, 3, 1 ). The chromosome may be despiralised with clear deeply stained R-bands remaining (Fig. I, 3, 2) and in these cells the 'early' X may also show R-banding although its condensation is normal. Thus, although both X's may be altered by the BrdU, it is still possible to tell them apart. If only one R-banded X chromosome was present this could be identified as the 'late' X because of the accompanying pattern of banding on other chromosomes, such as the elongated bands on 13q, several regions on chromosome 4, the segment near the centromere on the short arms

of chromosome 3, and the despiralised C-band on number 1. The other secondary constrictions, on 9 and 16, seem to be slightly less reliable, and in individuals where there was a large difference in size of C-band between the homologues it was noticed that these areas were asynchronous, possibly as the material in the larger band takes longer to replicate. Various X chromosome staining patterns are seen in Fig. I, 3, 2.

The sequence of DNA synthesis within the X chromosome, previously reported from autoradiographic studies, was also confirmed since the long arms may be R-banded and elongated while the short arms are unaffected and show normal G-banding. This pattern arises because the long arms finish replication later than the short arms.

I also found it was possible to reverse this procedure by growing the cells in medium containing BrdU and washing out the analogue before labelling with 20 μM thymidine in late S. This gave accentuated G-banding as the late-labelled regions contained more thymidine and were more deeply staining with Giemsa (Fig. I, 3, 3a). In this situation the 'late' X chromosome can have various patterns according to the time of labelling but often shows very pronounced dots at the sites of the G-bands on the short arms, and totally dark-stained long arms, so that the positive labelling is of the same sites seen in autoradiographs of cells labelled with tritiated thymidine (Fig. I, 3, 3b). The very clear differences between chromosomes

Fig. I, 3, 2.

FPG staining patterns of the late-replicating X chromosome, in four cells from a culture treated with BrdU during the last 7hours of incubation. The variable state of contraction of the "late" X, and the complex banding patterns of all the chromosomes are illustrated.

Top left. The entire "late" X has taken up BrdU and is pale-staining, while the other chromosomes have a segmented pattern of underspiralisation.

Bottom right. This cell was at a slightly more advanced stage of the S phase when the BrdU was added, and the "late" X is R-banded, while the secondary constrictions of chromosomes 1, 9, and 13 and also 3 and 4 are conspicuous, and some other chromosomes show R-banding.

Top right. Slightly later still; the "late" X is pale except for the centric regions, terminal band of the short arm, and a subterminal band on the long arm.

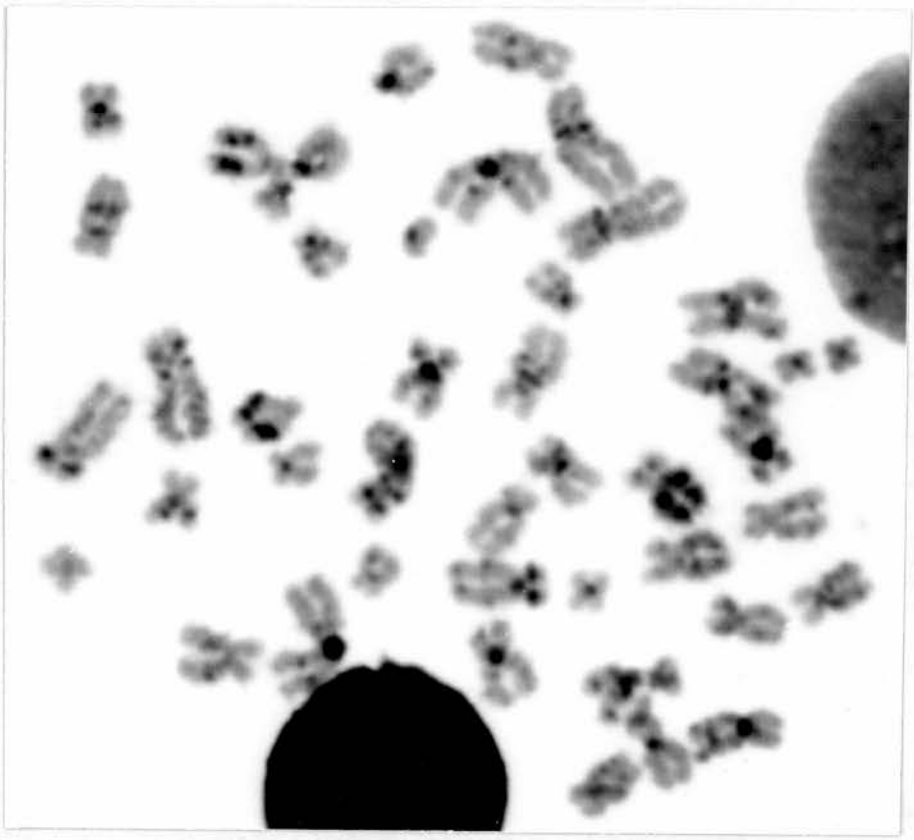
Bottom left. Only the medial band of the short arm of the X and most of the long arm were still replicating when the BrdU was added. (The long arm is more late-replicating than the short arm.) The secondary constrictions of chromosomes 1 and 13 are still conspicuous.

Fig. I, 3, 3a.

This human lymphocyte was grown for almost one complete cell cycle in the presence of BrdU, after which the analogue was washed out and replaced with thymidine during the last 5 hours of incubation. Intensely staining regions (FPG technique) contain more thymidine than the gray regions, i.e they are late-replicating. These are the distal segments of 13q, the secondary constrictions of chromosomes 1, 9 and 16, the short arms and centromeric region of chromosome 15, and the medial band of the short arms of chromosome 4, and the long arm of the "late" X, besides minor sites.

Fig. I, 3, 3b.

Autoradiograph of a cell after late-labelling with $^{3}$H-thymidine, showing the lack of resolution compared with the BrdU/FPG techniques illustrated in figures I, 3, 2 and I, 3, 3a.



4 and 5 were notable, chromosome 4 having two 'late' regions on the long arms as well as the one in the middle of the short arms, while chromosome 5 has only the short arm "late" region, although these chromosomes do not differ markedly by Q- or G-banding.

The FPG technique is, therefore, a very sensitive method for detection of DNA replication sequence, and substantiates the results on tritium-labelled chromosomes while providing a much less laborious procedure for very high resolution investigations of the DNA synthesis patterns of chromosome bands, and suggests that the bands are units of replication at the gross level although they almost certainly contain multiple subunits. It is possible that the analogue itself might affect the order of replication so as to produce an abnormal sequence, but the correspondence of the BrdU patterns with those produced by tritium labelling suggests that this is unlikely.

The BrdU and thymidine labelling techniques have been used in several comprehensive studies of replication patterns (Dutrillaux et al., 1973; Dutrillaux and Lejeune, 1975; Greschik et al., 1975; Epplen et al., 1975; Latt, 1975; Latt, 1976; Willard and Latt, 1976).

## B. ASYMMETRICAL C-BANDS

In 'harlequin' preparations I had noticed that the Y chromosome often stained indistinctly with no differentiation between chromatids and poorly defined morphology of the long arms. After the description of lateral asymmetry of H 33258 fluorescence in the centric heterochromatin of mouse cells following a single round of BrdU incorporation (Lin et al., 1974), and the less marked differentiation in the human Y chromosome in M1 cells (Latt et al., 1974), I examined both mouse (Fig. I, 3, 4) and human (Fig. I, 3, 5) M1 cells stained by the FPG technique.

The chromosomes in these M1 cells show overall gray staining with varying degrees of clarity of G-bands in addition to C-bands (Fig. I, 3, 5). When seen in juxtaposition to M2 cells, the M1 chromosomes are generally of the same staining intensity as the dark (unifilarly substituted) chromatid, but the state of contraction of the chromosomes leads to wide variations in the shades of staining. Asymmetrical C-bands, or bands within which sister chromatids show different staining levels in M1 chromosomes, are most easily detected in preparations where G-bands have been abolished by allowing the slides to age in the laboratory for at least four days before staining, and by giving longer light and 2 x SSC treatment than for M2 'harlequin' preparations. The results are also much more satisfactory in cells that have been treated

Fig. I, 3, 4.

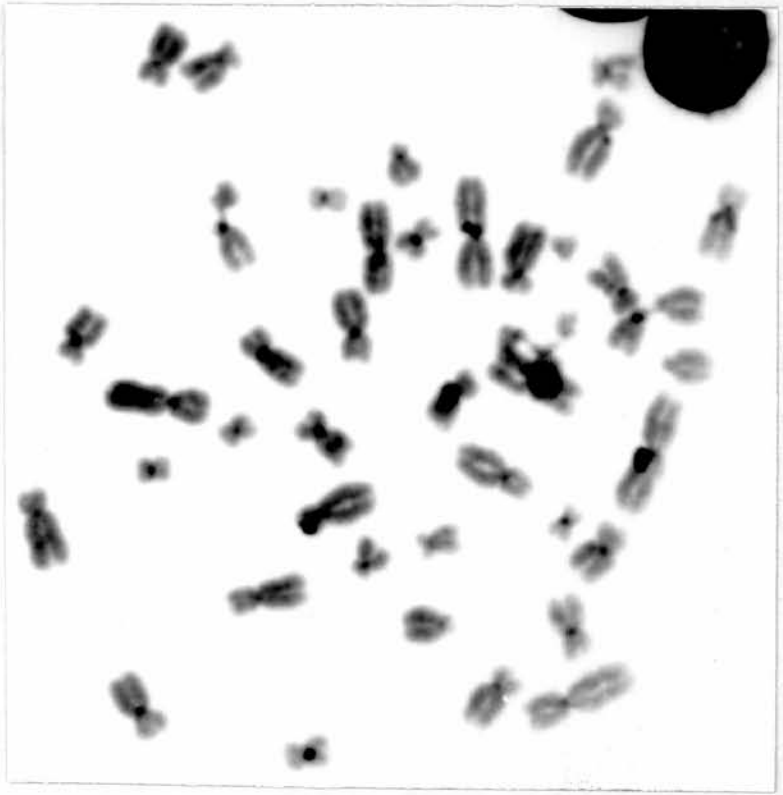
"Asymmetrical C-bands." Mouse (A9 cell line) chromosomes stained by the FPG technique after one round of BrdU incorporation . There is marked differentiation between chromatids only within the C-band areas, and in the metacentric chromosomes the contralateral arrangement of the darkly staining "beads" on opposite sides of the centromeres, is striking. One submetacentric chromosome (arrow) has a C-band showing complex asymmetry.

Fig. I, 3, 5a.

Human asymmetrical C-bands. Human lymphocyte chromosomes stained by the FPG technique after one cell cycle in the psesence of BrdU. Asymmetry is visible within the C-bands of chromosomes 15, 16 and Y, and one chromosome 1 shows simple asymmetry, while its homologue has a complex C-band.

Fig. I, 3, 5b.

Similarly treated cell showing simple asymmetry of staining in the C- band of a 1qh+ variant chromosome, and complex asymmetry of the other chromosome 1.



with higher concentrations of BrdU, preferably 100 μM or more. In preparations showing G-banding, a dark band across the whole of the C-band region is seen in chromosomes 1 and 16, and the secondary constriction of chromosome 9 is totally pale staining. However, a range of staining effects was seen, often even on the same slide, and good asymmetrical C-bands were sometimes seen in cells where enough G-banding remained for identification of the chromosomes involved.

i. Y Chromosome

Asymmetry was clearly seen in the C-band region of the Y chromosome (Fig. I, 3, 6) and particularly in longer Y chromosomes where extra material has been added to this C-band region. This explained the unusual staining of the Y chromosome in M2 cells, as chromosomes derived from the lighter M1 chromatid had completely pale C-band areas, and only half of the M2 cells had normal 'harlequin' Y's. The asymmetry in M1 cells is not confined to the Y chromosome, but is clearly seen in the C-band regions of chromosomes 1, 15 and 16 (Figs. I, 3, 5 and 6) and can also be detected in the smaller C-bands of chromosomes 4, 5, 17, 20, 21 and 22.

ii. Chromosome 16

This chromosome had very distinct asymmetry in all the cells examined (Fig. I, 3, 5 and 6) and here as well as in chromosome 1 and in the B, D, E, F and G group chromosomes there was often a perceptible pale region on

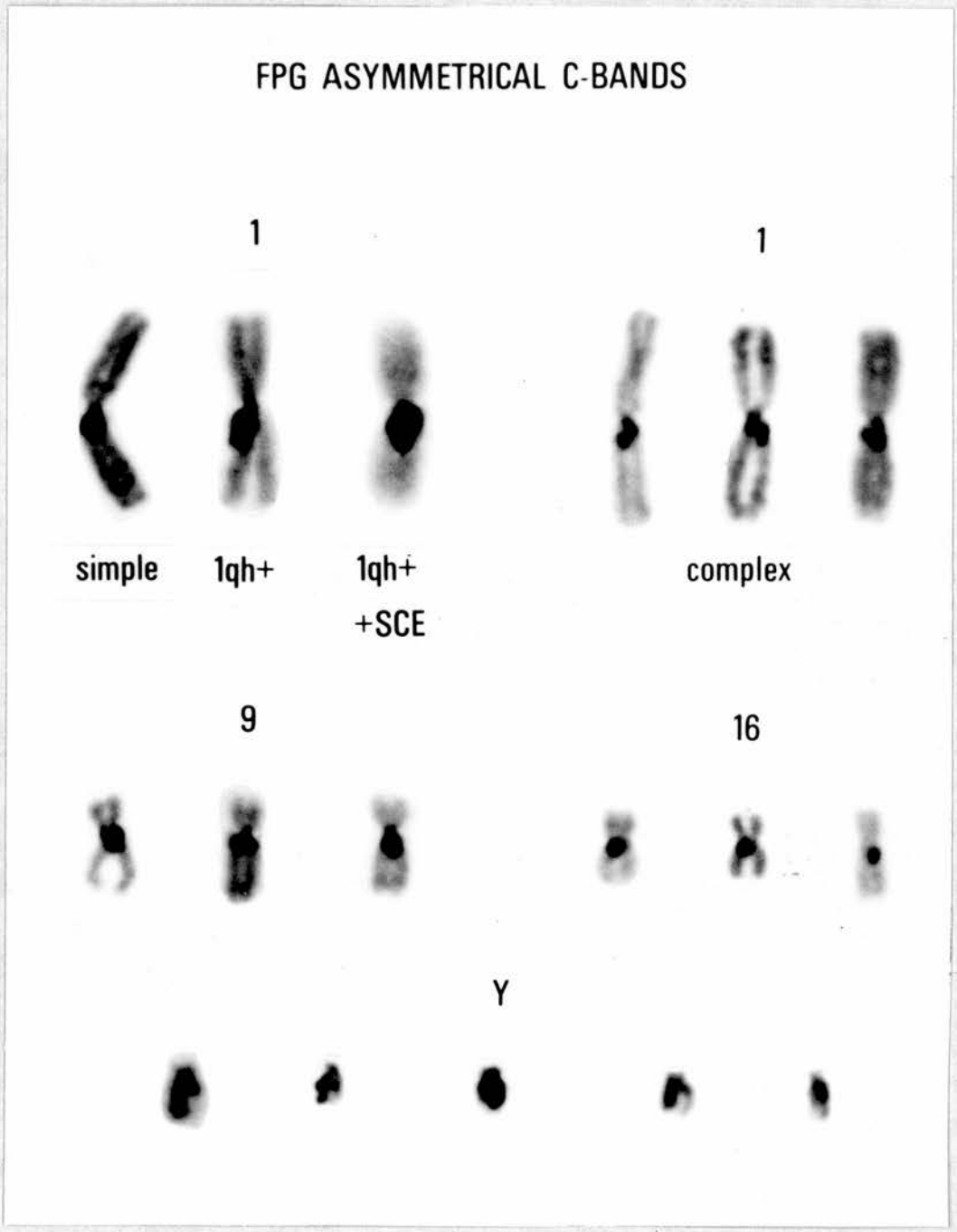



Fig. I, 3, 6.

Asymmetrical C-bands of some human chromosomes stained by the FPG technique after one cell cycle in the presence of BrdU.

the chromatid opposite the black dot of the C-band of the sister chromatid, as if a small section of 'harlequin' chromosome had been included in the otherwise undistinguished gray chromosome. Two individuals from different families carrying a variant chromosome 16 with a large C-band (16 qh+) were examined, and the extra material had been added on the same chromatid, and in some cells was visible as an extra black dot or bead.

iii. Chromosome 1

On first examination it seemed that only 60 to 70% of all the number 1 chromosomes examined showed C-band asymmetry. However, Angell and Jacobs (1975) reported that in some individuals there was compound asymmetry, with dark dots on both chromatids in the C-band area, but arranged diagonally with pale staining regions directly opposite each dark dot (Fig. I, 3, 6). It then became clear that asymmetry was apparent in every chromosome 1 examined provided the technique had worked (here the reliable staining pattern of chromosome 16 could be used as an indicator). This complex asymmetry appears to be another polymorphism, with a constant pattern in the cells of an individual. I have found that at least 10 cells from a subject must be examined to establish their C-band type, as the chromosome is often twisted in this region, perhaps because of the BrdU itself, and since the centromere has its own central black dot, a chromosome with simple asymmetry may be scored as complex in some cells where the centric dot is mistaken for part of a C-band.

A simple band may also be composed of more than one dot on the same chromatid, and when these are very small and arranged diagonally on one chromatid they are probably an indication of an underlying gyre in the chromatin, as this sort of pattern is also seen in G-banded cells, and on the dark chromatid of poorly fixed 'harlequin' chromosomes. The majority of the individuals I have now investigated have compound asymmetry of at least one homologue, since 29 individuals had two complex homologues; 47 individuals had one complex homologue; and five individuals had two simple homologues.

The staining pattern shows considerable diversity among individuals, as the dots may be of apparently different sizes and at varying distances from the centromere and from each other. Occasionally the complex C-band includes a third dot, although out of more than 80 individuals I have not seen a C-band composed of any more than three dots or 'beads'.

The area of the C-band seen by FPG staining seems to be less than that of the C-band produced by BaOH pretreatment, and with the FPG technique a distinct light staining band across both chromatids can often be distinguished between the centromere and the first bead. This size difference may, however, be due to staining artefacts, since in some of the BaOH treated cells the C-band is also composed of several beads, on both chromatids, and in these cases the total area is less that that of a band where the stain has 'spread' to give one block of C-band material.

In two unrelated individuals with a very large C-band of chromosome 1 (1 qh+), the asymmetry was of the simple type (Figs. I, 3, 5 and 6) and the long dark band appeared to comprise three large beads, all on the same chromatid except for the occasional SCE (see below, Section C). In smaller simple bands there may be one bead of varying size, or two beads.

iv. Chromosome 9

The asymmetry of staining appears in those regions of the karyotype that are now known to be the sites of satellite DNA localisation (see Appendix I and Chapter Three C). It was surprising at first that although chromosome 9 is a major hybridisation site for satellite DNAs (Saunders et al., 1972; Jones et al., 1973; Gosden et al., 1975a), it did not appear to show asymmetry, but the band in fact seems to be composed of a cluster of small beads (Figs. I, 3, 5 and 6) and is probably an extreme case of compound asymmetry, although this is only discernible in a small proportion of cells. In the individuals examined who have a 9 qh+ variant, the material is also added in a complex manner.

v. Other Chromosomes

Clear asymmetry was also seen in chromosomes 4, 5, 15, 21 and 22, where it was also seen in the chromosomal satellites. None of these regions is large enough to show compound asymmetry, and all of the sites are known to contain some satellite DNA.

vi. Asymmetrical C-bands of Other Species

a. Mouse. The clear lateral asymmetry of the C-bands of the acrocentrics and diagonal arrangement of the stained segments on either side of the centromeres in the metacentric chromosomes of the A9 mouse cell line, are dramatically demonstrated by the FPG technique (Fig. I, 3, 4). Complex asymmetry is seen on the longer arms of one submetacentric chromosome in this photograph (Fig. I, 3, 4), but this is not seen in all the cells and may, in fact, be a SCE occurring during the first cell cycle in BrdU, within the asymmetrical C-band.

b. Sheep. C-bands of sheep chromosomes are unusual in their pale Giemsa staining reaction (Schnedl, 1973) and these regions have an unstained 'empty' appearance after FPG treatment, so that no asymmetry is visible. This may in part be due to the fact that the satellite DNA known to be in the centric areas (A. Mitchell, personal communication) has a high GC content and probably does not separate into distinct bands of differing buoyant density in alkaline Caesium Chloride gradients (Curtain et al., 1973).

vii. Autoradiography and Asymmetrical C-bands

An attempt was made to demonstrate asymmetrical incorporation of $^{3}$H-BrdU or $^{3}$H-thymidine in M1 cells of human and mouse, but the resolution of ARG technique was not sufficient to distinguish differences in grain clustering between two halves of a C-band.

## C. DISTRIBUTION OF SCE

Due to difficulty in obtaining 'harlequin' chromosomes that also showed satisfactory banding patterns, and also because assignment of breaks to bands involves a large amount of observer error (Buckton, 1976), a thorough study of SCE distribution with respect to G-bands was not carried out. For the purposes of mapping SCE, chromosome arms were divided into equal sections designated q1, q2 and q3, and p1, p2 and p3 in addition to a very small region immediately on either side of the centromeres.

### i. Distribution of SCE between Chromosomes

This was shown to follow closely the pattern expected if SCE occur with frequency proportional to the metaphase lengths of the chromosomes (Appendix II). To the data in this paper (Appendix II) have been added the more recently scored SCEs, and Table I, 3, 2 illustrates the result pooled from the 11 individuals scored in Appendix II, plus a further 10 individuals. The reduced frequency of SCE in chromosome 16 and in the E, F and G group may simply be due to the scoring difficulties presented by these small chromosomes, since even with the high resolution of the FPG technique some small SCEs will be missed. The impression that the number of SCEs in the Y chromosome is low, is borne out by a comparison of the data from female G group chromosomes with those for the male G group, plus the Y chromosome. The Y could not always be identified, and due to the fact that in half the cells it is not

TABLE I 3 2

Percentage of SCE occurring in each chromosome or chromosome group.

| | 1 | 2 | 3 | B | C (3) | D | 16 | E | F | G (4) | G+Y |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ob. (1) | 8.3 | 9.1 | 7.0 | 14.9 | 37.5 | 11.3 | 2.8 | 4.6 | 2.2 | 2.1 | 2.6 |
| Ex. (2) | 8.4 | 8.0 | 6.8 | 12.4 | 38.7 | 10.7 | 3.4 | 6.2 | 5.2 | 3.9 | 6.1 |

1. Data from 6166 SCE from 21 individuals.
2. Data derived from measurements of chromosome lengths by Lubs et al, 1972.
3. The average length shown for the C group chromosomes is a mean of the total lengths for male and female C group chromosomes including the X chromosomes.
4. The observed SCE in the G group chromosomes were treated separately for males and females, so that the score for SCE in the G group for females is taken as a percentage of the 2762 SCE scored in female cells, and that for the male G group plus the Y chromosome is worked out using the total of 3404 SCE scored in male cells.

'harlequin' stained, some SCEs that might occur in this chromosome would be missed, so that the observed incidence of SCE in the Y may be spuriously low. In addition, the number of SCEs in the female group G chromosomes may be exaggerated due to the presence in one female of a chromosome 22 with long short arms and prominent satellites, that facilitated the detection of SCE between satellites and centromere. All but one of the SCEs seen in this region were found in preparations from this individual.

Numbers of SCE per chromosome:- The numbers of chromosomes with 0, 1, 2 ..... n SCE show a good fit to a Poisson distribution, providing further evidence that, in general, SCEs occur at random throughout the complement. There is no indication that SCEs interfere in the manner of chiasmata at meiosis (reviewed by John and Lewis, 1965).

ii. Distribution of SCE within Chromosomes

The distribution of SCE is shown in Appendix II (Fig. 3) and in Fig. I, 3, 7, where the data on a further 3133 SCEs have been added to the published figures. The distribution obtained from the pooled data does not differ greatly from that in the Appendix for the first 11 individuals, except for the number of SCEs in the middle section of the short arm of the C-group chromosomes, where the pooled data reveal a much greater frequency of SCEs.

In chromosomes and groups B, C and 16, and the long arms of 2, D and E, the middle sections of the arms seem

Fig. I, 3, 7.

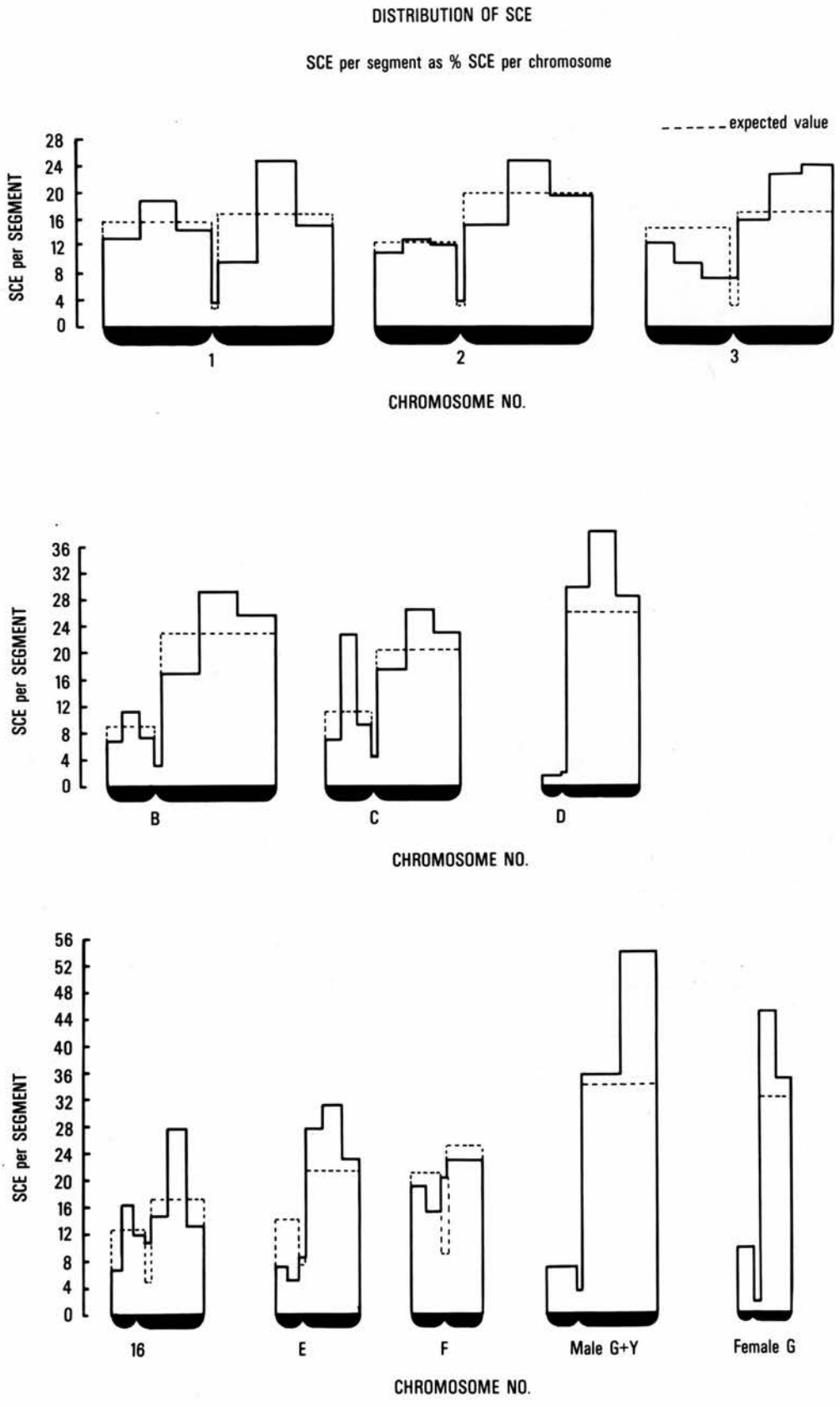

DISTRIBUTION OF SCE
SCE per segment as % SCE per chromosome
expected value
SCE per SEGMENT
28
24
20
16
12
8
4
0
1
2
3
CHROMOSOME NO.
SCE per SEGMENT
36
32
28
24
20
16
12
8
4
0
B
C
D
CHROMOSOME NO.
SCE per SEGMENT
56
52
48
44
40
36
32
28
24
20
16
12
8
4
0
16
E
F
Male G+Y
Female G
CHROMOSOME NO.

TABLE I 3 3

a. Position of SCE in metacentric chromosomes where long and short arms could not be distinguished.

| Segment / Chromosome | p/q 1 | p/q 2 | p/q 3 |
|---|---|---|---|
| 3 | 52 | 72 | 53 |
| F group | 22 | 35 | |

b. Proportion of total SCE assigned to each segment : without (i), and with (ii) inclusion of excess SCE from (a), (above).

| | | Proportion of SCE in each segment (%) | | | | | | | Total SCE |
|---|---|---|---|---|---|---|---|---|---|
| | | p3 | p2 | p1 | c | q1 | q2 | q3 | |
| 3 | i | 12.5 | 9.7 | 7.4 | 7.4 | 15.95 | 22.8 | 24 | 257 |
| | ii | 13.5 | 14.1 | 10.4 | 4.4 | 15.4 | 21.9 | 20.4 | 434 |
| F | i | | 19 | 15.2 | 20.3 | 22.8 | 22.8 | | 79 |
| | ii | | 23.9 | 16.9 | 11.8 | 21.3 | 26.1 | | 136 |

to have the highest proportion of SCEs. This may in part be scoring bias, and there may be an underestimate of the numbers of SCEs close to the telomeres, since these regions frequently have an equivocal grayish appearance and I tended to score a doubtful SCE as negative. It is possible that small exchanges in these regions are below the resolution of the FPG technique.

It is clear that there is no excess of SCE in the secondary constrictions of chromosomes 1 and 16, and although few SCEs were positively assigned to the Y chromosome, the long arms of the (G + Y) group in males do have a higher frequency of SCEs than the G group in females. The data in Appendix II (Fig. 3) included counts on two individuals with unusually large Y chromosomes, but the inclusion of a substantial amount of data from males with average Y chromosomes has not altered the 'profile' of this group (Fig. I, 3, 7). The distribution profile for SCE in chromosome 3 and in the F group may be false, since it was not always possible to distinguish between the long and short arms of these metacentric chromosomes. This meant that a fairly large proportion of SCE (177 out of 434 for chromosome 3, and 57 out of 136 for the F group) are not included in the figures plotted in Fig. I, 3, 7. There is no reason to suppose that either short or long arm SCEs are being lost from the data preferentially, so that in Table I, 3,3, the excess SCE for each segment not definitely assigned (Table I, 3, 3a) have been divided between the appropriate sections of the long and short

arms (Table I, 3, 3b). The distribution is altered by the inclusion of these figures to give a higher proportion of SCE in the middle segments of 3p and 3q, and in the distal segments of both arms of the F group chromosomes. The low number of SCEs in the short arms of the E group may again be due to the difficulty of scoring SCE in this very short segment.

a. Frequency of SCE at Centromeres. In M2 cells it was difficult at first to distinguish SCEs occurring at the centromere itself from twists in the chromosome, so that in the first series of mapping (Appendix II) ambiguous chromosomes were not scored and the number of centromeric SCEs was low. This rarity was confirmed by counting SCEs in M3 cells where a chromosome derived from a centric SCE has only one remaining darkly stained arm (Fig. I, 4, 1). I found only 5% of the SCEs were of this type (Appendix II), but since the publication of those results I have scored a large number of cells and found the proportion of centric SCE to be much higher, with an average frequency of about 12%.

Since the later results are from cells exposed to 25 μM BrdU instead of the 160 μM used in the earlier experiments (Appendix II), it seemed there might be some sort of saturation effect on the numbers occurring at the centromere, so that a proportion of up to 15% at 25 μM BrdU was reduced by the time the overall SCE frequency reached the level seen at 160 μM BrdU, due to a higher frequency of SCE occurring elsewhere. This possibility

TABLE I 3 4

Frequency of centromeric SCE scored in M3 cells.

| Conc. BrdU (μM) | Chromosomes scored | SCE | Centric SCE | % Centric SCE |
|---|---|---|---|---|
| 25 | 4821 | 925 | 106 | 11.5 |
| 25 | 1708 | 433 | 27 | 6.2 |
| 50 | 800 | 174 | 13 | 7.5 |
| 100 | 351 | 81 | 11 | 13.6 |
| 150-160 | 2770 | 905 | 77 | 8.5 |

TABLE I 3 5

Comparison of proportion of centric SCE scored in M2 and M3 cells from the same cultures.

| | M2 | M3 |
|---|---|---|
| SCE | 245 | 117 |
| Chromosomes | 1881 | 870 |
| % Centric SCE | 13 | 13.4 |

TABLE I 3 6

Positions of Centric SCE.

| Chromosome | 1 | 2 | 3 | B | C | D | 16 | E | F | G | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Observed | 77 | 53 | 51 | 74 | 372 | 28 | 71 | 33 | 32 | 8 | 799 |
| Expected | 35 | 35 | 35 | 70 | 278 | 104 | 35 | 70 | 70 | 70 | |
| Ratio O:E | 2.2 | 1.5 | 1.5 | 1 | 1.3 | 0.23 | 2 | 0.5 | 0.5 | 0.1 | |

was checked and shown to be without grounds (Table I, 3, 4); it was clear that variation amongst cultures and scoring methods could give rise to large differences in numbers of centric SCEs, especially if only low numbers of cells were examined. The results in Table I, 3, 4 show a large difference between the frequencies of centric SCE scored in two experiments using 25 μM BrdU. This variation was also illustrated by comparing the frequency of centric SCE scored in identically treated duplicate cultures of the same blood sample; 10 cells were scored from each culture and in one case there were 13% centric SCEs compared with only 8% in the second culture. The data in Table I, 3, 5 show that if M2 and M3 cells are scored on the same slide, the number of centric SCEs seen at M2 is a very good estimate of the actual number detected at M3, so that centric SCEs can after all be accurately distinguished from twists, using the FPG technique. The overall range of proportions of centric SCE is from about 6 to 15%.

1. Positions of centric SCE. Of all the SCEs scored at the centromere itself, the chromosomes involved were noted for a total of 799 SCE (Table I, 3, 6). The results show that the D and G group are strikingly under-represented, but these are acrocentric chromosomes in which SCEs are not easily distinguished. If satellites are pronounced, occasionally SCEs may be seen between these and the centromere, but these SCEs may be occurring much more frequently than we can detect. The E and F group chromosomes have fewer SCEs overall so their lack of centric SCEs may again be an observation problem. The B and C group have numbers

TABLE I 3 7.

SCE in C-bands, scored at M2.

| | | Control | Mutagens | Total |
|---|---|---|---|---|
| SCE | Females | 1037 | 10079 | 11116 |
| | Males | 2088 | 30421 | 32509 |
| Cells | Females | 83 | 97 | 180 |
| | Males | 233 | 790 | 1023 |
| SCE in C-bands as % total SCE. * | 1 | 0.26 | 0.04 | 0.05 |
| | 9 | 0.03 | 0.03 | 0.03 |
| | 16 | 0.19 | 0.04 | 0.05 |
| | Y | 0 | 0.03 | 0.03 |
| Chromosomes with SCE in C- bands as % chromosomes of the same type. | 1 | 1.27 | 0.7 | 1.7 |
| | 9 | 0.16 | 0.55 | 0.9 |
| | 16 | 0.95 | 0.85 | 1.75 |
| | Y | 0 | 1.39 | 1.08 |

* Values for the Y chromosome are calculated as a percentage of the total SCE for male cells only.

close to the expected frequency, while chromosomes 2 and 3 have slightly higher numbers.

The doubling of the expected frequencies in chromosome pairs 1 and 16 is interesting as the centromere itself is separate from the centric heterochromatin as distinguished by the FPG technique. This high frequency of SCE may therefore be due to the presence of a junction between the heterochromatin and euchromatin near the centromere (see below).

b. Frequency of SCE in C-bands.

The profiles in Fig. I, 3, 7 show a marked decrease in SCE frequency in the C-band region of chromosome 1 ($1q_1$) and a smaller depression in the corresponding region of chromosome 16. Table I, 3, 7 shows numbers of SCEs that occurred immediately distal (1, 9 and 16) or proximal (Y) to the C-band. The results are pooled from five individuals and scored in cells treated with 25 μM BrdU only, or with various concentrations of Mitomycin C, Ethyl Methane Sulphonate or Adriamycin (see Chapter Five). The lack of SCE in the C-band areas is more complex than it appears and the arrangement of material in these specialised areas seems to have a marked effect on SCE frequency.

Chromosome 1. In M1 cells the asymmetrical C-bands described above are composed of one or more dots or beads. In complex C-bands these beads are arranged on sister chromatids at different loci. In two unrelated individuals having the 1 qh+ variant, all the dark staining material in M1 cells

was on one chromatid (Figs. I, 3, 5 and 6) but SCEs were seen occasionally in these regions (Table I, 3, 8). These SCEs always appear to occur in between the beads (Fig. I, 3, 8).

Using 25 μM BrdU we expect about four to five SCEs per cell cycle in the whole cell, so that in the 111 M1 cells examined from individual B, about 500 SCEs could have occurred. Assuming that the 1 qh+ C-band is roughly equivalent to chromosome 21 in size, it should have about 1.9% of the SCEs in this area if the exchanges are distributed randomly according to metaphase length. Thus, the expected number of SCEs in the asymmetrical C-band is 9.5, and is very close to the observed number for individual B, despite the fact that the C-band area contains a great deal more DNA than does chromosome 21, since the heterochromatin is greatly compacted. However, since SCEs seem to occur only between beads, the frequency of SCEs in these very restricted junction regions must be very high considering the length of the material involved. In individual A the frequency of SCE is much lower than expected on the same criteria unless all the ambiguous SCEs are counted as positives, so that no firm conclusion can be drawn as to the behaviour of the asymmetrical C-bands in all individuals. The areas of the normal C-bands are too small to score SCE within the C-bands at M1. In complex C-bands, SCE would result in simple C-bands, and may explain the occasional difficulty of classification when a particular homologue is not unequivocally 'complex' in all cells. Similarly,

SCEs in simple C-bands would give rise to a few 'complex' C-bands.

In M2 cells, the SCEs in the C-bands seem to occur most frequently at the junction of the band with the remainder of the chromatid (Fig. I, 3, 8). A complex C-band in M2 cells is still visible as a very dark bead on the black chromatid, and a corresponding grayish bead on the paler chromatid, and chromosomes are occasionally seen (Fig. I,3,8) where an SCE must have occurred immediately proximal or distal to the FPG C-band. The numbers are too small to say whether C-band SCE are more frequent in individuals with two complex homologues of chromosome 1.

Chromosome 9. The C-band area of this chromosome in M2 cells frequently stains lightly across the whole chromosome as in G-banded preparations. Thus, it is not usually possible to say whether SCEs have occurred within the area or at the interface of this region with the rest of the long arm or just below the centromere (Fig. I, 3, 8).

Chromosome 16. SCEs within the C-band of chromosome 16 are rare, and I have not seen any such exchanges in cells from individuals with a large C-band on 16, where there were two beads on one chromatid. The SCE scored in this chromosome appeared to be at the C-band/long-arm interface.

Y chromosome. The C-band of a large Y seems to be composed of more than one band in Quinacrine- or ASG-stained preparations. The rare SCEs seen in the Y were at the C-band interface, and only on two occasions did I see SCEs in the

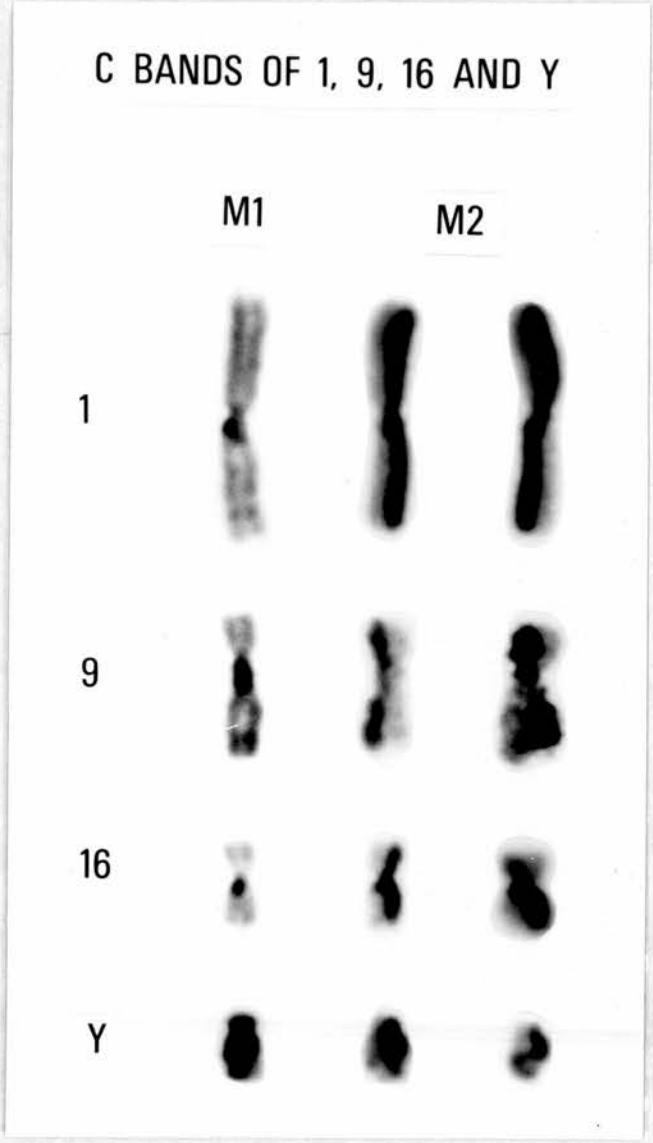



Fig. I, 3, 8.

The left and centre columns show C-bands of chromosomes stained by the FPG technique, in M1 and M2 cells respectively, while the chromosomes on the right illustrate SCE in, or at the interface of, the C-bands.

TABLE I 3 8.

Frequency of SCE in asymmetrical C-bands of 1qh+ chromosomes scored in M1 cells.

| Individual | Conc. BrdU (uM) | No. of chromosomes scored | - SCE | + SCE | Possible SCE | % of chromosomes positive |
|---|---|---|---|---|---|---|
| A | 25 | 204 | 190 | 7 | 7 | 3.4 |
| | 100 | 29 | 23 | 1 | 5 | 3.4 |
| B | 25 | 111 | 98 | 9 | 4 | 8.1 |

middle of the C-band, and it is quite possible that these had occurred between beads, although this could not be seen in the cells concerned (Fig. I, 3, 8).

c. SCE in relation to G-bands. The appearance of G-banded 'harlequins' does not facilitate exact localisation of exchange points (Fig. I, 2, 3). However, I had the impression that the SCEs were occurring in pale bands, or at the interface of dark and pale bands. This is an impression to be treated with care since the observation of a dark band will encourage the viewer to assign the break to the adjacent pale band, although it is possible that a small part of the dark band may be missing. One hundred SCEs were plotted according to bands, in M2 and M3 cells but this is not a comprehensive study. All the SCEs in every cell were not plotted, so there is a large selection bias, as the bands are often most clear in particular chromosomes, such as the distinctive pairs 1, 11, 12 and 7. For a proportion of the SCEs it was not clear whether the break was at a dark-light interface or in the adjacent band. If all these SCEs are added in to the 'interface' category the results are as follows:

| | |
|---|---|
| Centric | 15 |
| Pale band | 17 |
| Interface | 52 |
| Dark band | 16 |

This is biased in favour of interface SCE. If the SCEs scored equivocally as 'Pale band OR pale/dark interface'

are added in to the pale category, and the 'Dark band OR interface' are added in to the dark band category, the result is the opposite extreme, and biased in favour of SCE in bands:

| | |
|---|---|
| Pale band | 28 |
| Dark band | 17 |
| Interface | 40 |
| Centric | 15 |
| | 100 |

Even with the result thus weighted against 'interface' SCEs, 40% are in this category.

The average of the above two distributions is:

| | |
|---|---|
| Pale band | 22.5 |
| Dark band | 16.25 |
| Interface | 46 |
| Centric | 15 |

These are very small numbers, since only 100 SCEs are scored, and some were not confidently assigned to any position, but the results do indicate a trend towards SCE occuring at junctions of light and dark band material. It is possible that those exchanges that do appear to be within the dark bands may in fact be in small strips of pale band type material since metaphase dark bands are composed of several prophase bands that have merged as the chromosomes condensed (Bigger and Savage, 1975).

# D. DISCUSSION

## i. Mechanism of Staining

The reaction of BrdU-substituted chromatin with dyes is very complicated. Originally the effects of BrdU on chromosome condensation were thought to be due to preferential uptake of the analogue into AT-rich DNA, but at concentrations of BrdU that are widely used there is no preferential incorporation into the repetitious DNA in mouse (Grady and Campbell, 1974) or rat cells (Schwartz and Kirsten, 1974). The latter authors found that at 100 μM BrdU, base substitution was very efficient with more than 90% of the thymine replaced in all types of DNA. Only at 0.1 μM BrdU - i.e. well below the concentrations used for late-labelling or 'harlequin' chromosomes - is uptake non-random, and here the base is more commonly taken up into repetitive and intermediate DNA fractions.

The quenching of H 33258 fluorescence in association with BrdU-substituted polymers in solution was thought to parallel the dullness seen in cytological preparations in more highly substituted chromatids (Latt, 1974), but is not an adequate explanation for the lighter staining with Giemsa. Harlequin chromosomes illustrate the pitfalls involved in the interpretation of Acridine Orange colour reactions, since the unifilarly substituted chromatid is yellow-green and the sister chromatid containing more BrdU is orange-red, a difference which might have been interpreted as differential denaturation of DNA, with single

stranded DNA in the dull chromatid (Bobrow et al., 1972b; Labs et al., 1973). In fact it is more likely that it is due to the altered conformation of the chromatin affecting the intercalation of the dye molecules. The differential Giemsa reaction with the FPG technique requires exposure of the chromosomes to light to produce a marked contrast, and Goto et al (1975) interpreted their results of Feulgen staining as an indication that extensive photolysis of BrdU-containing DNA was occurring on the slides; however, Comings and Avelino (1975) have suggested that this Feulgen differentiation is more likely to be due to diffuseness of the chromatin rather than loss of intact DNA.

The effects of altered DNA-protein interactions must be the most important factor in the altered stainability of the chromatids, and there is evidence that BrdU-substituted DNA is more tightly bound to protein (Gordon et al., 1973; Lapeyre and Bekhor, 1974; David et al., 1974) although this was not confirmed by Simpson and Seale (1974) who did, however, agree that the conformation of such chromatin is altered by BrdU substitution. It is interesting that both chromatids of harlequins may retain their G-banded pattern, before this is abolished by ageing the slides or over-exposing them to light. Similarly, bands are seen in AO stained chromosomes before the differentiation between chromatids 'develops' under exposure to UV light. I have also found that the two chromatids of harlequin

chromosomes have differing sensitivity to trypsin, since the pale chromatid shows bands after shorter digestion times than does the dark chromatid.

My experiments on combinations of techniques to obtain G- or R-bands in addition to harlequins show that once the FPG technique has been applied, the R-banding procedure (Hot 1M $NaH_2PO_4$ at low pH) will not alter the staining although the same treatment will reverse the pattern of G-bands produced by ASG. The same chromatid therefore appears always to be the dully fluorescent or pale-Giemsa-staining chromatid after a variety of cytochemical treatments. It is interesting that the harlequins are produced by the C-banding-like technique of Korenberg and Freedlender (1974) since hot alkali removes a large amount of DNA from the chromosomes (Comings et al., 1973) yet heat and salt treatment of the harlequin FPG technique does not remove much DNA from the doubly-substituted chromatid (Dutrillaux, cited by Comings and Avelino, 1975).

It seems therefore to be the conformation of the chromatin that is important (Brodie et al, 1975) and Comings (1975a) thinks that since H 33258 does not intercalate but binds to the outside of the DNA double helix, possibly by hydrophobic reaction, this dye has more opportunity for base specific reaction and is more sensitive to base composition than fluorochromes such as Quinacrine. This suggests a reason for the sensitivity

of H 33258 fluorescence to BrdU substitution, compared with Quinacrine which is a poor stain for harlequin chromosomes, but it does not explain the effectiveness of AO, which is thought to intercalate in much the same way as Quinacrine.

A small piece of evidence that conformation is the most important factor in Giemsa stainability is the occurrence, albeit in only one of nearly 9,500 cells examined by G-banding, of a spontaneously underspiralised X chromosome in a routine blood culture. This chromosome had the same pale, elongated appearance with R-bands as that of a BrdU substituted chromosome.

ii. Conclusions on Late Replication

The sensitivity of the BrdU labelling technique, or pulse labelling with thymidine of BrdU-substituted chromosomes, as methods for detection of DNA replication, is being widely exploited both to confirm and extend studies on the normal patterns of DNA synthesis and to compare replication sequences in different tissues. For example, tritium labelling studies of DNA synthesis patterns in fibroblasts and amniotic cells had suggested that the sequences in these tissues were not identical (German and Aronian, 1971) but the new BrdU technique has been used to show that there is no difference detectable by this higher resolution technique (Epplen et al., 1975). The accuracy of the technique even revealed slight asynchrony between homologues (Greschick et al., 1975)

and this method is a convenient alternative to autoradiography in studies of X chromosomes (Dutrillaux et al., 1973) and investigations of inactivation patterns in abnormal, supernumerary, or translocation chromosomes (Mikkelsen, 1976; Latt, 1974a; Latt et al., 1976). The results in general lend support to the hypothesis that there is multifocal control of replication initiation and completion, and that structural bands may also be identified as units of replication at the cytological level, probably containing many small units.

iii. Asymmetrical C-bands

It is intriguing that the first report of lateral asymmetry appeared in 1967, when Huang was examining the effects of BrdU and other mutagens on rat chromosomes (Huang, 1967). He saw asymmetrical staining of the heterochromatic region of the rat X chromosome and even noticed SCE, but was unable to explain the odd appearance of this chromosome. When Lin et al (1974) reported the lateral asymmetry of BrdU-substituted mouse heterochromatin in M1 cells, they suggested this differentiation was due to the known marked difference in thymine (T) content between the strands of the satellite DNA (45% and 22%; Flamm et al., 1967). The C-band asymmetry seen in human chromosomes also occurs in areas known to contain satellite DNAs (Gosden et al., 1975a). There are four known human satellites, comprising only a small proportion of the human genome (less than 5%) compared with the 10% satellite DNA in mouse. The differences in T content found by strand separation in alkaline Caesium Chloride

gradient centrifugation in human satellite DNAs are small, with a 10% difference for human satellie I (Schildkraut and Mayo, 1969), and only about 3% difference between the strands of satellite II (A. Mitchell, personal communication).

Table I, 3, 9 illustrates the staining properties and satellite DNA contents of the C-band regions for chromosomes 1, 9, 16 and the Y, with mouse C-bands for comparison. From this Table it is clear that chromosome 16 is the odd one out on Giemsa 11 staining, besides containing exclusively satellite II (as far as can be detected). Apart from the difference after ASG treatment, chromosome 16 behaves most like the mouse C-bands, yet the clear FPG C-band asymmetry in 16 is surprising if strand bias in T content is the only factor involved, since mouse satellite DNA has a 23% difference between strands while the human satellite II has only a 3% difference, although small differences in base composition and arrangement can have very marked effects on conformation and on DNA-protein interactions. However, it should be noted that this region may contain highly repetitious DNA sequences other than those identified as satellite DNAs (Gosden et al., 1975b) and DNA of this type may well be involved in staining asymmetry (see below). Like mouse C-bands, the secondary constriction area of human chromosome 9 is negatively stained by ASG and Quinacrine, yet it has the most dramatic G-11 reaction with a large, intense G-11 band, while mouse C-bands stain only faintly by this method.

TABLE I 39.

Staining properties and Satellite DNA content of major C-bands of human and mouse.

| | | 1 | 9 | 16 | Y | Mouse |
|---|---|---|---|---|---|---|
| Giemsa | ASG | + | - | + | + | - |
| | C-bands | + | + | + | + | - |
| | G-11 | + | +++ | - | + | - |
| Fluor-escence. | Q | - | - | - | +++ | - |
| | H 33258 | + | + | + | + | + |
| | FPG | + | + | + | + | + |
| BrdU + H 33258 | | +A* | + | +A | +A | +A |
| BrdU + FPG | | +A | + | +A | +A | +A |
| Satellite DNA. ** | I | | +++ | | +++ | |
| | II | +++ | +++ | ++ | +++ | |
| | III | | +++ | | +++ | |
| | IV | ++ | +++ | | +++ | |
| Mouse satellite DNA | | | | | | +++ |

*A denotes asymmetrical staining

** results of Gosden et al, 1975a.

Obviously there is more involved in the staining reactions than satellite DNA content alone. The material stained by various techniques (Table I, 3, 9) may not be identical in each case, and it may be important that the FPG C-banded areas of human chromosomes appear smaller than the BaOH C-bands. This has been confirmed by measurements in kangaroo rat cells (Bostock and Christie, 1976). The Quinacrine-intense region of the Y chromosome may also differ from the BaOH C-band, and Souder and Laraya (1976) have observed a deleted Y chromosome where there is no intense Q fluorescence, yet a small C-band remains.

It is most likely that the stainability is dictated by the conformation of these regions of the chromatin, and the underlying base composition will have a bearing on this tertiary structure, but the same DNA type may behave in various ways in its several locations, possibly due to the influence of other associated or adjacent DNA sequences. For instance, although satellite II is on chromosomes 1, 9, 16 and the Y, asymmetry is not seen on the 9, possibly due to the presence of other material. Chromosomes 9 and Y seem to have all four satellites, yet they differ in several properties, notably their Quinacrine fluorescence. Satellite DNAs of one class may also evolve in different ways in the different sites, so that satellite III, for instance, may not have identical sequences on chromosomes 1 and 9. The satellite DNAs so far recognised comprise less than half the total highly repetitive DNA, and other

repetitive sequences are known, such as the ribosomal RNA genes in the nucleolar organiser regions, and the 5S RNA genes on chromosome 1 (Steffensen et al., 1974; Atwood et al., 1975). A class of repetitive DNA that is specific to males has recently been isolated (Cooke, 1976), and studies in this Unit have shown by in situ hybridisation that this material is on the Y chromosome, and not in the distal part of the Quinacrine-intense region (C.J. Bostock and A. Mitchell, in preparation). This DNA constitutes a large proportion of the total Y DNA and may be involved in the singular cytological properties of the Y chromosome.

The functions of heterochromatin and of satellite DNAs are still very much in question. Since closely related species may have widely differing satellite DNA contents it is unlikely that large amounts of this material are necessary for vital functions such as homologue recognition for pairing at meiosis, and such specific mechanisms as this could not rely totally on satellite DNA as it is too widespread in the genome. The satellite DNA is probably not transcribed, as Flamm et al (1969) were unable to find any RNA complementary to mouse satellite DNA, and Southern (1970) has shown that the base sequences of guinea pig satellite would generate nonsense or repeating dipeptides. Postulated functions for satellite or repetitive DNAs included roles in folding of chromatin, recognition of centromeres and initiation of replication, besides the possession of regulatory genes,

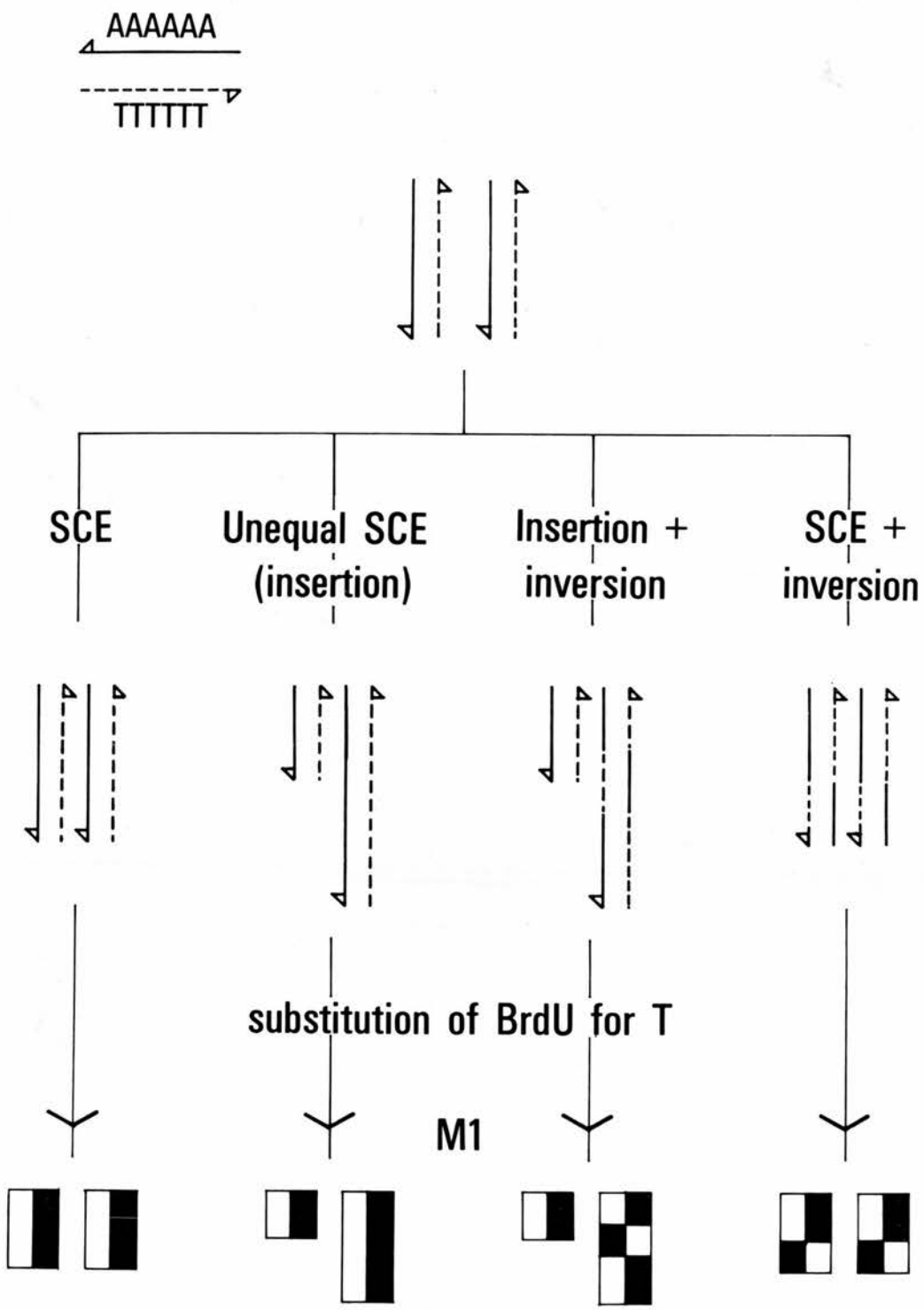
multiple copies of certain genes, or the ability to form recombination sites, or to act as raw material for evolution to work upon - an attractive theory for which there is no real evidence (Walker et al., 1969; Bostock 1971). However, no conclusions can so far be drawn due to lack of proof, but by investigating the composition and behaviour of these DNAs some clues as to their function may be found.

Unequal crossing-over has been proposed as a mechanism by which multiplication of repeated sequences may occur (Southern, 1975; Smith, 1976) at the molecular level, and at the cytological level as the means by which, for instance, a large Y with two intensely fluorescent Q-bands could arise (Sperling and Lackmann, 1971). Crossing-over between two sets of repeated sequences at different loci could be the mechanism of Robertsonian translocation. We may be seeing the results of this sort of process in the human asymmetrical C-bands. Crossing-over at meiosis in plants and insects probably does not occur in heterochromatin (reviewed by John and Lewis, 1965) but in Drosophila mitotic crossing-over does occur frequently in heterochromatic regions (reviewed by Brown, 1966). The initial results on SCE localisation suggested that these did not occur regularly in constitutive heterochromatin (Fig. I, 3,7) but examination of M1 cells showed that SCE could occur in the asymmetrical C-bands, but only between 'beads' (Fig. I, 3, 8). This may be the mechanism by which the complex patterns of C-band asymmetry and the variations on size of the bands may arise.

In the C-band of chromosome 1 an insertion of similar material from one chromatid onto the other would lead to a large C-band of simple asymmetry with two beads, while insertion along with an inversion would lead to complex asymmetry (Fig. I, 3, 9). This, in effect unequal, crossing-over might be expected to happen only very rarely, and there is no evidence for unequal crossing-over from SCE studies in M2 and M3 cells, but the asymmetrical C-bands with their unusual DNA content and structure may be exceptions where unequal cross-over can occur because of the high probability of exchange at the interfaces of 'beads'. It is easier to envisage this process at the level of a small DNA sequence or a gene locus, such as the unequal crossing-over known to occur between sister chromatids at 'bar' (Peterson and Laughnan, 1963) and 'bobbed' loci (Schalet, 1969) in Drosophila, but difficult to imagine this occurring in such large stretches of DNA as the regions in question in the human genome. The larger C-bands must take exceedingly long periods to evolve, and the chances of this occurring repeatedly to form the 1 qh+ C-band of three or four 'beads' are low enough to make this variant quite rare. Due to variable methods of ascertainment and criteria for classification, the frequency of 'variants' of the secondary constriction of chromosome 1 varies widely among reports (Lubs and Ruddle, 1971; Nielsen et al., 1974; Craig-Holmes et al., 1973) while in 952 individuals examined in this Laboratory 3.5% large variants (large C-bands) were identified, of which only 0.22% of the total were in the

Fig. I, 3, 9.

# Derivation of Asymmetrical C bands



'very large' category (Buckton et al., 1976). Very small C-bands were also reported to be rare in these studies, and the general finding is that most chromosomes belong to the intermediate C-band size classes. By treating human fibroblast cultures with the cross-linking agent Mitomycin-C, Hoehn and Martin (1972) found that they could induce a new variant chromosome 1 with an increased C-band size. This occurred in one out of 10 cultures and was present in all 42 cells examined, but had never been detected in preparations treated with other agents such as mitotic-arresting substances or Sendai virus. It would be of interest to examine the asymmetrical C-bands in Mitomycin-C-treated chromosomes for unequal crossing-over and consequent generation of new sizes of C-bands (Fig. I, 3, 9).

The Y chromosomes examined so far have never shown complex asymmetry and the large Y found in two brothers is clearly of the 'simple' type. This material looks different from the C-band regions of 1 and 16 as, instead of the C-band having a clear black and white striped appearance, the pale chromatid of the Y often appears as a diffuse grayish region with no clearly defined edge (Fig, I, 3, 5 and 6). The C-band of chromosome 9 often appears to be composed of four to six beads (Fig. I, 3, 6) and extra material is also added in a complex manner, unlike the 1 qh+ variants. It is interesting that the C-band of 9 undergoes considerable variation such as pericentric inversion as well as size polymorphism (see, for example, Madan and Bobrow, 1974) with the breaks occurring preferentially

in the heterochromatin or at the junction with euchromatin (Hansmann, 1976). Pericentric inversions of chromosomes 1 and 16 are probably much less common (see Buckton et al., 1976).

Human chromosomes have only a small proportion of C-band material, but the asymmetry of C-bands has also been dramatically demonstrated in the X chromosome of the Indian Muntjac (Carrano and Wolff, 1975) and in the kangaroo rat Dipodymus Ordii (Bostock and Christie, 1976). The precise localisation of the satellite DNAs of the kangaroo rat had previously been demonstrated by in situ hybridisation, and the degree of thymine bias was known for each satellite, so that Bostock and Christie (1976) were able to show that the same satellite could be associated with asymmetry in one location, but with lack of stainability by the FPG technique elsewhere in the karyotype. This confirms the conclusions from my studies on human chromosomes that other factors besides thymine bias are involved in differential staining in the C-bands and that possibly other DNA sequences and conformation of chromatin determine the staining properties.

The asymmetrical C-bands thus afford greater insight into the arrangement of chromatin and its behaviour, and should also tell us a great deal about the mechanisms of chromosome rearrangements and development of polymorphisms in evolution.

iv. Location of Exchange Points

The vexed question of the function and properties of bands and the reasons behind their apparent differential susceptibilities to alteration or breakage by specific agents is being investigated from several angles. The distribution of BrdU-induced SCE, like that of X-ray induced aberrations, fits a Poisson distribution so that each chromosome has roughly the number of breaks expected for its metaphase length, but several studies on chromosome and chromatid aberrations induced by X-rays have provided evidence for a preferential localisation of these break points in the pale G- or Q-bands (Caspersson et al., 1972; Holmberg and Jonasson, 1973; Seabright, 1973a; San Roman and Bobrow, 1973;) and it seemed possible that the DNA in these regions, being less condensed and possibly less well 'protected' in interphase, might be more available to undergo breakage and exchange. Savage et al (1976) exposed cells to the alkylating agent Quinacrine Mustard instead of X-rays, and still found that nearly 80% of the breaks were in the pale ASG bands. Since QM interacts more strongly at metaphase with the Q-positive bands, the damage might be expected to show localisation to these regions rather than to the 'interbands', and while most breaks did not follow this prediction a 'reasonable proportion' (22% of the breaks) occurred in the dark bands, so that the situation was not straightforward. A prepond-erance of breaks induced by Nitrogen Mustard has also been found in the pale Q-bands (R. Howell, 1974; unpublished results). Recent data on X-ray induced breaks mapped by

using G-and R-banding sequentially on the same cells, have given a more reliable picture of the distribution of breaks by avoiding some of the observer error involved in assigning breaks to bands (Buckton, 1976). It was concluded that 30% of the breaks were at the interface of dark and pale bands, and this is interesting in view of the impression gained from my SCE observations that the exchanges here are also occurring at junctions of bands and at heterochromatin/ euchromatin interfaces. Smyth's study (Smyth and Evans, 1976) of tritium-labelled chromosomes showed a trend towards localisation of SCE in the dark G-bands and in chromosomes with a higher porportion of Giemsa-intense material. This may be due to increased chances of damage due to the higher concentration of the isotope, in the more condensed areas of the chromatin.

In all these studies on X-rays and alkylating agents, and in my BrdU SCE studies, it is interesting that there is no apparent preference for breakage in heterochromatin, although chromatid aberrations due to larger doses of BrdU are known to be concentrated in these regions (Hsu and Somers, 1961; Kaback et al., 1964). However, Natarajan and Klasterska (1975) found more SCEs in the constitutive heterochromatin of the X chromosomes in the vole Microtis Agrestis, and previously Sugiyama (1971) had noted frequent SCE in the heterochromatic regions of rat chromosomes. It was surprising that Schnedl et al (1976) also found an unusually high rate of SCE in the human facultatively heterochromatic X. The inactive X was identified by tritium labelling, but Schnedl et al were able to rule

out the objection that the excess SCEs were tritium-induced by also scoring SCEs in cells that had not been treated with isotope, where one of the X chromosomes (identified by Q-banding prior to H 33258 staining) still had consistently more SCEs than its homologue. In constitutive heterochromatin it is concievable that the exchanges are occurring in small euchromatic regions, or at junctions analagous to the ones seen between beads in human and kangaroo rat chromosomes, but the inactive X results cannot be explained in this way.

There were conflicting data from autoradiographic studies on the frequency of centromeric SCE, since there seemed to be a low frequency in wallaby chromosomes (Geard, 1974), but a level of 25% in kangaroo rat cells scored at M3 (Gibson and Prescott, 1974). Studies of _in vitro_ X-ray induced chromosome rearrangements (Buckton, 1976), showing 30% of the breaks in centromeres and telomeres correspond well with the results for constitutional rearrangements (Jacobs _et al._, 1974). Nineteen percent of these breaks were at the centromeres, a result which compares favourably with the figure of up to 15% centric SCE found here, so that this specialised region seems to be very susceptible to involvement in aberrations, possibly because of the juxtaposition of centric heterochromatin. Also cells carrying rearrangements of this type may be more viable than those with other exchanges, if less vital genes are located near the centromeres so that cells can withstand their loss or mutation.

The most interesting finding of all these studies on breakage of chromosomes seems to be that there is something very unusual about the junctions between heterochromatin and euchromatin, or of condensed and less compact DNA.

# CHAPTER FOUR

## APPLICATION OF THE FPG TECHNIQUE TO INVESTIGATION OF SCE AND CHROMOSOME BEHAVIOUR IN NORMAL CELLS

### A. i. Segregation of Chromatids

Although results from autoradiographic studies suggested that segregation of chromatids to daughter cells at mitosis was random (reviewed by Wolff and Heddle, 1968) some experiments were thought to show that in cells in their second cycle after labelling, chromatin containing 'parental' DNA was completely separated from that containing 'grand-parental' DNA (Lark et al., 1966). The clear labelling patterns of FPG-stained chromosomes allow this to be tested, and I have examined the easily distinguished chromosome pairs 1, 2 and 16 in M3 cells (Fig. I, 4, 1).

If segregation of chromatids from M2 harlequin chromosomes is random, M3 cells with the following patterns,

a. Two harlequin homologues,
b. One harlequin and one completely pale-stained homologue,
c. Both homologues completely pale,

should occur in the proportions a:b:c = 1:2:1. The

patterns are disturbed to some extent by SCE but chromosomes can still be easily classified into these categories as shown in Table I, 4, 1. A chromatid that is darkly stained along more than half its length is scored as dark, and the same procedure was followed for pale chromatids, so that the errors involved should cancel each other. However, all chromosomes with only one dark arm remaining as a result of a centric SCE were scored as harlequins, so that there are too many cells in the first two columns in Table I, 4, 1i (categories a and b). Despite these considerations the proportions observed of 23% : 52% : 25% are very close to the 1:2:1 ratio expected if segregation is random.

For the G group chromosomes the observed frequencies of cells with four pale chromosomes, four harlequin chromosomes and all the intermediate combinations are shown in Table I, 4, 1ii. In this situation the ratio of dark to pale chromatids should be 1:3 and is in fact about 1:3.9, so that there appear to be too many pale staining chromatids. This implies a loss of cells where G group chromosomes are derived from the grand-parental DNA, so that they still have a darkly stained chromatid. The cells examined are from females to avoid the complicating factor of Y asymmetry. However, the numbers are not large, so that the figures for the G group are not good evidence against random segregation, while the results for chromosomes 1, 2 and 16 show convincingly that assortment of chromatids is random.

TABLE I 4 1.

i. Segregation of Chromatids.

| Chromosome | | a | b | c |
|---|---|---|---|---|
| 1 | No. | 52 | 113 | 40 |
| | % | 25.4 | 55.1 | 19.5 |
| 2 | No. | 17 | 42 | 23 |
| | % | 20.7 | 51.2 | 28.1 |
| 16 | No. | 40 | 93 | 54 |
| | % | 21.4 | 49.7 | 28.9 |
| Total | No. | 109 | 248 | 117 |
| | % | 23 | 52 | 25 |
| Expected | % | 25 | 50 | 25 |

ii. G Group chromosomes.

| Cell type | 4p | 3p1h | 2p2h | 1p3h | 4h |
|---|---|---|---|---|---|
| Observed | 26 | 45 | 37 | 28 | 9 |
| Expected | 18 | 36 | 36 | 36 | 18 |

p;- pale stained chromosome

h;- "Harlequin-stained" chromosome

ii. Segregation of Asymmetrical C-bands

The black beads of asymmetrical C-bands are clearly visible on the dark chromatids of M2 chromosomes, so that the segregation of these areas can also be followed. The results are described in Appendix I. Half of the FPG-C-band areas can be expected to stain lightly across both chromatids at M2, and this is the case for the human Y and for mouse A9 cell line chromosomes. The issue is complicated in the case of human chromosome 1 by its complex asymmetry, but the proportions of chromosomes with a dark bead remaining, or with an all-pale C-band, were examined in individuals with compound asymmetry of one or both homologues and shown to fit the expected distribution (Appendix I, Table 3).

Some complications arise due to variations in staining intensity, since the amount of BrdU taken up by human chromosomes may be reduced in second and subsequent S phases compared with the initial replication phase. This is clearly seen in a proportion of M3 cells where there is three-way staining differentiation of chromosomes (Fig. I, 4,1), and the most newly synthesised chromatids are of a gray tone intermediate between the dark and pale chromatids, (Fig. I, 4, 1), suggesting that the new DNA is less fully substituted with BrdU. Similarly, the black C-band beads in M2 cells may be opposite a gray bead clearly visible on the pale chromatid, and alternatively an expected pale bead on the dark chromatid may appear gray. This accounts

Fig. I, 4, 1.

Human lymphocyte stained by the FPG technique after three cell cycles in the presence of BrdU, showing three-way differentiation . About one quarter of the material in the cell still contains unsubstituted DNA and so stains darkly with Giemsa, while the bifilarly-substituted chromatin is pale-staining. The most recently synthesised chromatin shows an intermediate gray-stained tone, possibly due to a reduction in BrdU incorporation with successive cell cycles.

for the high number of cells where chromosome 16 still appeared to have a darkly staining FPG half C-band (Appendix I, Table 3).

The behaviour of chromosome 9 is once again unusual. The secondary constriction is often completely pale in M2 cells, possibly because of G-banding, but may also appear to be grayer than the very insubstantial area seen in ASG preparations. Occasionally a gray : white differentiation between chromatids within the C-bands is visible (Fig. I, 3, 8), but no consistent pattern and no striking black beads of the type seen on chromosomes 1 and 16 are found.

## B. EVIDENCE FOR AND AGAINST SPONTANEOUS SCE

### i. BrdU dose Response

At very low concentrations of BrdU the SCE frequency in cells of a Chinese Hamster (CHO) cell line has been shown to rise steeply from the lowest level at 0.25 μM to about 1 μM, and then to rise more slowly to a plateau level at about 20 μM (Wolff and Perry, 1974). SCEs are not easily discernible at very low BrdU incorporation levels, but several experiments on blood lymphocytes were carried out using concentrations of BrdU from 5 to 200 μM (Table I, 4, 2). It is clear that the numbers of SCEs continue to rise from about 0.17 per chromosome (7.7 per cell) at 5 μM BrdU to about 0.35 per chromosome (16.1 per cell) at 200 μM BrdU.

### ii. 'Background' SCE Frequency and Growth Rates

Most of the subsequent experiments were carried out using 25 μM BrdU as this gave reliable results. The background SCE level at this concentration is about 10 SCE per cell or 0.22 per chromosome, with the actual number per cell ranging from about three to 30. The fairly wide range of SCE frequencies among cultures and individuals may be mainly due to differences in incorporation of BrdU, although fairly similar levels of SCE in cultures from the same individual carried out on three occasions at widely spaced intervals are shown in Table I, 4,3. In conclusion, differences of several SCEs per cell on average

TABLE I 4 2.

SCE:- BrdU dose response.

| Conc. BrdU (μM) | 5 | 10 | 25 | 50 | 100 | 150 | 200 |
|---|---|---|---|---|---|---|---|
| Ave. SCE/ chromosome | 0.168 | 0.176 | 0.202 | 0.245 | 0.272 | 0.290 | 0.350 |
| Standard Error | 0.008 | 0.010 | 0.010 | 0.010 | 0.013 | 0.013 | 0.034 |

TABLE I 4 3.

SCE frequencies in repeat cultures from one individual (25μM BrdU).

| Culture | 1 | 2 | 3 |
|---|---|---|---|
| SCE | 429 | 188 | 182 |
| Chromosomes | 2277 | 1162 | 1007 |
| Ave. SCE/ chromosome | 0.188 | 0.162 | 0.181 |
| Ave. SCE/ cell | 8.65 | 7.4 | 8.3 |

may be expected as a result of culture variation and cannot be attributed to more significant causes.

a. Effects of FUdR. The use of 5-Fluorodeoxyuridine (FUdR) as a thymidylate synthetase inhibitor to facilitate the uptake of BrdU was tested. FUdR (0.4 μM) and Uridine (6 μM) were present throughout the culture period. The mitotic index was depressed compared with cultures containing BrdU only, and there were fewer M3 and more M1 cells, so that growth seemed to be slower. Table I, 4, 4 shows that the SCE frequency at 25 μM is slightly increased in the presence of FUdR (11.3 compared with 9.9 per cell) while this effect is more marked at 10 μM BrdU where the overall increase in the presence of FUdR is from 7.2 to 11.9 per cell. Because of these effects, FUdR was not used routinely, since substitution with BrdU was quite adequate to give clear harlequins without added FUdR, although it is possible that the use of FUdR might lead to more reproducible results as it reduces the variation in BrdU uptake among individual cells by suppressing thymidine metabolism in all the cells.

Table I, 4, 5 shows the proportions of M1, M2 and M3 cells in three day cultures from seven individuals and illustrates the wide variations in growth rates. This does not seem to be consistently affected by the age of the subject, although cultures from newborn babies do tend to have high M3 frequencies, often up to 90% of the cells after 69-72 hours of incubation, and also more M2 cells after 48 hour incubation periods.

TABLE I 4 4.

Effect of FUdR (0.4 μM) on Frequency of SCE.

| Individual | | 1 | 2 | (1+2) | 3 |
|---|---|---|---|---|---|
| Conc. BrdU (μM) | | 10 | 10 | 10 | 25 |
| Ave. SCE/Cell (No. of cells) | +FUdR | 10.7 (20) | 11.9 (25) | 11.4 (45) | 11.3 (51) |
| | -FUdR | 7.2 (21) | 7.2 (26) | 7.2 (47) | 9.9 (50) |

TABLE I 4 5.

Proportions of first, second and third metaphase cells in three- day cultures.

| Individual | No. of Cells | Percent | | |
|---|---|---|---|---|
| | | M1 | M2 | M3 |
| 1 | 334 | 11 | 22 | 67 |
| 2 | 181 | 22 | 31 | 47 |
| 3 | 200 | 33 | 48 | 19 |
| 4 | 71 | 25 | 59 | 16 |
| 5 | 259 | 34 | 36 | 30 |
| 6 | 239 | 21 | 38 | 41 |
| 7 | 107 | 44 | 40 | 16 |
| Average (7) | 1391 | 27 | 39 | 34 |

iii. SCE in a Ring Chromosome

A single monocentric ring chromosome can give rise to a double-sized dicentric ring, and it is thought that this results from sister chromatid exchange between ring chromatids. The studies by Brewen and Peacock (1969a) on a C-group ring chromosome showed that it formed dicentric rings spontaneously and this this frequency was increased by the incorporation of tritiated thymidine, although the rate of dicentric formation did not seem to be further affected by the level of isotope incorporation. This latter observation may be due to the saturation effect later demonstrated for all tritium induced SCE, but there is full documentation of the fact that constitutional rings do form dicentrics spontaneously (for example, see Lejeune et al., 1968; Pathak and Sinha, 1972).

I was able to examine the behaviour of a ring chromosome 14 in a baby born in Edinburgh, and in her mother who proved to be carrying this chromosome. It was also of interest to examine the SCE frequency in these individuals. The results of chromosome analyses on the baby and her mother are shown in Table I, 4, 6. It is clear that the ring is fairly stable, with a low number of cells containing a dicentric ring, although up to 10% of the cells in both mother and baby have lost the ring. This may occur as a result of non-disjunction after formation of a dicentric ring, so that SCE may be occurring more frequently in the ring chromosomes than we can see. The apparent frequency of SCE in the ring in cultures that had not been treated

TABLE I 4 6.

Chromosome analysis on baby and mother with 45,r14 karyotype.

| Analysis | Baby | | | Mother | | |
|---|---|---|---|---|---|---|
| | M2 | M3 | 0* | M2 | M3 | 0* |
| 46+ring | 100 | 86 | 90 | 25 | 41 | 85 |
| 45-ring | 7 | 8 | 2 | 3 | 3 | 10 |
| 46 normal | | | 5 | | | 3 |
| 46,dic ring | 2 | 2 | 1 | | 2 | |
| 46, 2 dic ring (interlocked) | | | | | | 1 |
| 47,+2rings | | | | | | 1 |
| 45,+ small ring | | 1 | 1 | | | |
| 45 + abn (Dp-) | | 1 | | 1 | | |
| 45 + pulverised chromatin | | | 1 | 1 | 3 | |
| Polyploid; | | | | | | |
| 2 dic rings | 1 | | | | | |
| no ring | | 2 | | | | |
| Total cells | 110 | 100 | 101 | 30 | 49 | 100 |

0*; routine 3-day culture, orcein analysis.

with BrdU, was one SCE in 100 cells in the baby, and nil in the mother, while in M2 cells after BrdU treatment the dicentric ring occurred three times in 110 cells from the baby and was not seen in thirty cells from the mother. At M3, SCE had apparently occurred in the ring in two out of 100 cells and two of 50 cells from the baby and mother respectively (Table I, 4, 6). The overall frequencies of SCE in both individuals at 25 μM BrdU are shown in Table I, 4, 7, along with those from three female newborn baby controls. It is interesting that the abnormal baby has a high frequency of SCE (16 SCE per cell at 25 μM BrdU), although it is still within the range for babies with no chromosome abnormalities, as normal male babies with frequencies of about 20-22 per cell (0.45 and 0.48 per chromosome) at 160 μM BrdU had been found (Appendix II, Table 1).

The ring chromosomes and a dicentric ring, stained by the FPG technique, are shown in Fig. I, 4, 2.

TABLE I 4 7.

SCE frequency in baby and mother with ring chromosome 14 and in three control babies.

| | Baby | Mother | Controls | | |
|---|---|---|---|---|---|
| | | | 1 | 2 | 3 |
| SCE[a] | 1723 | 239 | 83 | 213 | 371 |
| Chromosomes | 4972 | 1209 | 487 | 1003 | 1533 |
| Ave. SCE/Cell | 16 | 9 | 8 | 9.8 | 11 |

a. 25μM BrdU

Fig. I, 4, 2a.

"Harlequin" chromosomes, showing a ring chromosome 14.

Fig. I, 4, 2b.

A dicentric ring with symmetrically opposed centromeres, in another cell from the same individual. This dicentric ring is though to have arisen as a result of SCE within the ring chromosome.

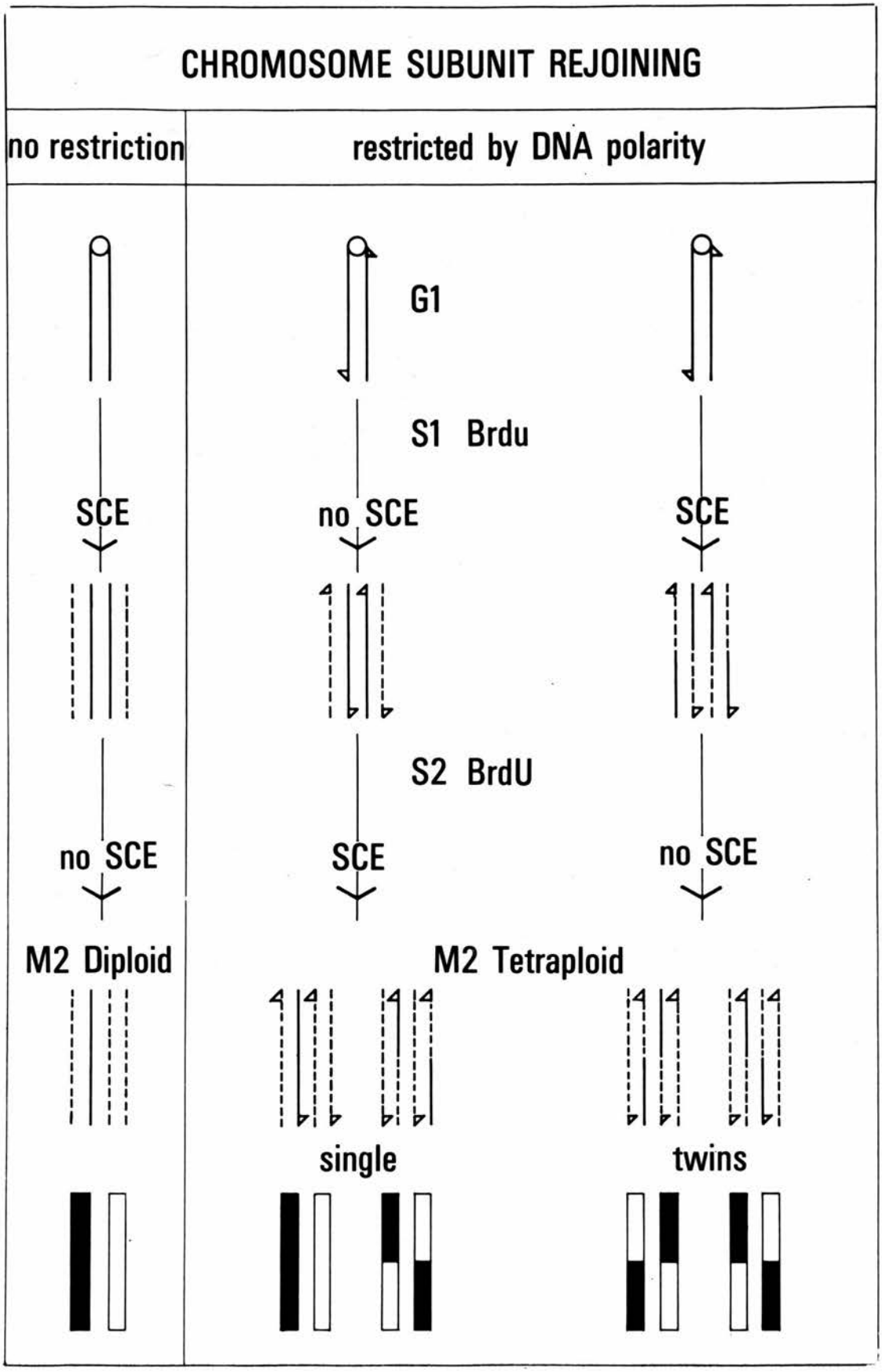
## C. RESTRICTED SUBUNIT REJOINING

The very fact that SCE may be observed in M2 cells suggests that there is restriction in the way chromosome subunits may rejoin, such as we would expect from a uni-neme model of chromosome structure where sister chromatids contain subunits of opposite polarities. In tetraploid M2 cells, all the SCEs that have occurred during two cell cycles can be seen, and chromosomes containing SCEs that occurred two cycles previously have been duplicated to give a pair of chromosomes with SCEs at identical sites in each ('twins'), while the SCEs that occurred in the cycle immediately prior to observation are represented only once ('singles'). If rejoining is in fact restricted the ratio of singles to twins should be 2:1, but although some auto-radiographic studies confirmed this, others produced widely varying results with ratios of up to 10:1 (see Chapter One).

### i. M3 Cells

In blood cultures tetraploid cells are rare, but another way to investigate this is to examine M3 cells and compare the numbers of SCEs that occurred during the first and second cycles of culture with those that occurred in the third cycle. The latter are visible as exchanges in complete harlequin chromosomes, while the results of earlier SCE are seen as chromosomes where only part of one arm is now darkly stained (Fig. I, 4, 1). If SCEs occur with equal frequency in each cell cycle the total score

Fig. I, 4, 3.


CHROMOSOME SUBUNIT REJOINING
no restriction
restricted by DNA polarity
G1
S1 Brdu
SCE
no SCE
SCE
S2 BrdU
no SCE
SCE
no SCE
M2 Diploid
M2 Tetraploid
single
twins

for the first two cycles should be double the score for the third cycle. However, half of the SCEs that occur in the third cycle are between pale stained chromatids and not visible, so that the ratio of cycles (1 + 2) to 3 should be 4:1. The actual ratio found was 1219:337, or about 3.6:1, in 200 M3 cells. This is fairly close to a 4:1 ratio and good evidence for restricted rejoining of chromatids and thus for the mononeme chromosome model (Fig. I, 4, 3). However, the fact that the ratio is lower than the expected 4:1 suggests that more SCEs are occurring in the third than in the first or second cycles, as we might expect from the increasing BrdU content of the chromosomes. A similar effect was found by Geard (1974) in tritium treated wallaby chromosomes.

ii. Endoreduplicated Cells

The occasional M2 endoreduplicated cells seen in blood cultures displayed clearly the strictly maintained spatial relationship of 'new' to 'old' chromatin in diplochromosomes (Fig. I, 4, 4) that had been described in ARGs by Walen (1965) and by Schwarzacher and Schnedl (1965). Except where the pattern is interrupted by SCE the paler staining chromatids are always on the outside of the darker ones. The reasons for this arrangement are not known, but the centromere does not seem to be crucial in this orientation as I have seen mutagen-induced acentric fragments behaving in exactly the same way as normal chromosomes - an observation in agreement with the results of Herreros and Gianelli (1967).

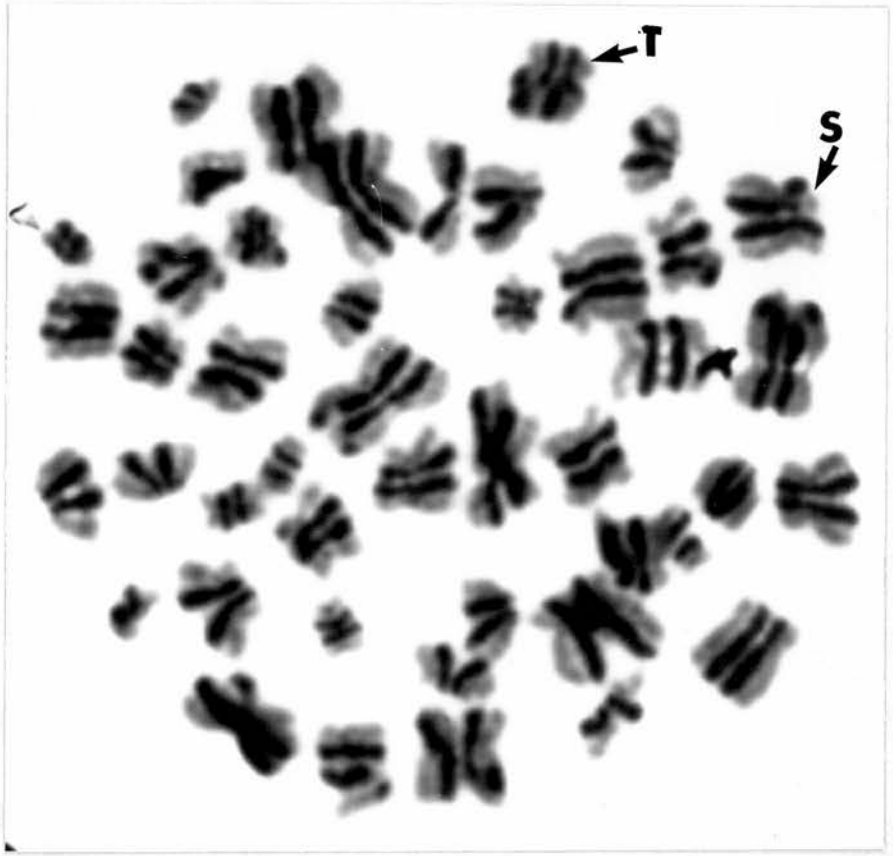



Fig. I, 4, 4.

An endoreduplicated human lymphocyte, showing "harlequin"staining. The newly synthesised chromatids are laid down in a characteristic manner on the outside of the darkly-staining parental chromatids, except where the pattern is interrupted by SCE. The arrows indicate twin (T) and single (S) exchanges.

In diplochromosomes the chromatids are often thin and tend to wind round each other so the SCEs are not easily scored in these cells. However, in the few cells that were examined, the numbers of singles and twins were 49 and 21. This is very close to a 2:1 ratio considering the small numbers. The lack of inter-chromosome exchanges I observed in the diplochromosomes is in accord with the results of Olivieri and Brewen (1966), who showed that SCEs were much more common than inter-chromosome exchanges, but that when the latter did arise after isotope incorporation they were usually between the two inner chromatids, suggesting that the diplochromosomes retain their close pairing in interphase. This non-random rejoining in diplochromosomes also indicated the restrictions on the fate of broken ends of chromatids.

The endoreduplicated cells I examined were mainly in cultures that had been treated with chemical mutagens (see Chapter Five), where there was a high SCE frequency yet I found only 58 SCEs out of 239 diplochromosomes - i.e. 0.12 per chromosome, or 5-6 per diploid cell - while the average for the slides was 13-25 SCEs per cell. In the one endoreduplicated cell from a control culture not treated with mutagens the rate of 15 SCEs per 46 diplochromosomes was about average for the culture. Scoring difficulties may contribute substantially to the low SCE frequency in these unusual cells, but it is an interesting observation and may be a peculiarity of their response to mutagens.

A low SCE level in endoreduplicated cells is not a general rule, however, since one cell in this study and the colcemide-induced endoreduplicated cells in CHO cultures (Wolff and Perry, 1975) did not have a reduced SCE frequency.

## D. DISCUSSION

### i. Random Segregation

The classical observations on semi-conservative replication and segregation of chromosomes (Taylor et al., 1957) which were followed by the demonstration of semi-conservative replication of the DNA itself (Meselson and Stahl, 1958) were questioned by Lark et al (1966) who presented evidence from mouse embryo and Chinese hamster cells labelled with tritium that segregation of old and newly synthesised chromatin to daughter cells at mitosis did not follow a random pattern. The conclusions were drawn from observations of label distribution over interphase nuclei presumed to be in a second cycle after labelling (Lark, 1966), but it is rather more reliable to look at metaphase chromosomes in cells where the labelling status is accurately known.

The clear identification of each generation of chromatin by its staining properties using the FPG technique enabled me to examine the proportions of dark- and light-staining chromatids in M3 cells and confirm that segregation of these chromatids, in colcemide treated blood cells at least, is random. H 33258 staining of anaphase chromosomes of the Muntjac (Mayron and Wise, 1976) has been used to demonstrate random segregation, and Fernandez Gomez et al (1975) have additional evidence from tritium labelling in non-colcemide treated meristem cells of Allium cepa, so that in conjunction with tritium-labelling results in several

cell types (reviewed by Wolff and Heddle, 1968), the evidence for random segregation of chromatids is conclusive. The segregation of asymmetrical C-bands has also been shown to be random in the present study.

ii. Ring Chromosome

In both individuals with the ring chromosome 14 (the baby and her mother) the dicentric was seen in one in 100 cells in the absence of BrdU, so that the results imply a very low rate of spontaneous SCE in this chromosome. If SCEs occur with a frequency proportional to metaphase chromosome length (see Chapter Three), we can expect a total of about 3.56% of all the SCEs to occur in chromosome 14. If SCE occurs at an average level of eight per cell per cell-cycle, 100 cells should have about 800 SCEs per cycle, of which 28 would be in chromosome 14. The ring chromosome 14 (Fig. I, 4, 2) studied here contains nearly all the material of a normal 14 chromosome, identified by both G- and R-banding (K. Buckton, personal communication), so it should undergo SCE in about 14 out of every 100 cells. This should result in the formation of 14 dicentric rings per 100 cells in each cell cycle, but since only one to three such rings are seen, there must be a reduction in SCE frequency, or a strong tendency of cells to lose the dicentric. Assuming that all the cells that have the karyotype 45XX, -r, had lost the ring as a result of non-disjunction of a dicentric ring, SCEs would have occurred in the ring in up to a further 10% of the cells so that the frequency would approach the expected level of 14%.

The observed frequency of dicentric rings is much lower than that in a C-group chromosome described by Brewen and Peacock (1969), where the frequency was 0.121 in 48 hour cultures and 0.116 at 72 hours - i.e. more than 10 times higher than for the ring 14 here. It is known that rings do vary widely in their rates of dicentric formation, or at least in the numbers that are detected, and three ring chromosomes of the D-group have been described by Lejeune et al (1968) where the dicentric was seen in nine out of 419 cells, four of 129 cells and only one of 208 cells from the three patients.

It is interesting that the proportion of dicentrics does not differ between the mother and baby for the ring 14, although the baby had a fairly high rate of SCE overall (about 16 per cell, compared with nine per cell in her mother). Although the cultures containing BrdU did have a higher proportion of dicentrics than the control cultures, the baby's cells had no more dicentrics at M3 than at M2 although there are probably more SCEs overall in the third cycle. The mother did have two dicentrics in 50 M3 cells compared with none out of 30 M2 cells. The frequency of ring loss seems slightly higher in the mother than in the baby in non-BrdU treated cultures, but on the other hand, more rings were lost in the baby's cells after BrdU treatment, an observation in line with the expectation that the higher rate of SCE in the baby might result in more dicentric formation and subsequent ring loss.

Pathak and Sinha (1972) found a highly variable ring that broke frequently to give an assortment of different sizes of rings and rod chromosomes. They therefore suggested that the rings arose by breakage and sister union rather than by SCE. In the case of the ring 14, I have only twice seen a small ring (Table I, 4, 6), although there are several cells with six D-group chromosomes where the ring must have broken, but the frequencies are not very high, and also the dicentrics always had symmetrically opposed centromeres as we expect in double rings that have been derived from monocentric rings by SCE rather than by breakage and sister union, so that SCE seems a reasonable explanation for my results.

iii. Spontaneity of SCE

The question of spontaneity of SCE is left open by the dose response curves for BrdU, since the data plotted for CHO chromosomes by Wolff and Perry (1974) can be extrapolated either to zero or to a low spontaneous rate, and the same is true for my data from blood lymphocytes. Kato's studies using AO fluorescence showed no increase in SCE with increased BrdU exposure at low concentrations of the analogue, and led him to conclude that all the SCEs in the cell line in question were spontaneous (Kato, 1974b). It should be noted that the D6 Chinese hamster cell line is unusual in its low background frequency of SCE; only two to three per cell at concentrations where human lymphocytes show six to nine, and CHO cells (Perry and Wolff, 1974) about 12 per cell.

The number of SCEs per cell cycle seemed to bear a relationship to cell DNA content, although the interpretation of comparisons of different studies is complicated by the use of various concentrations of BrdU and isotope. However, apart from the D6 Chinese hamster cell line, human, Chinese hamster, mouse, wallaby, kangaroo rat and rat kangaroo all have similar SCE frequencies *in vitro* and Kihlman and Kronborg (1975) also found a SCE level *in Vicia* that was consistent with its high DNA content. DNA content is by no means the only factor governing SCE frequency since cells from individuals with Bloom's syndrome have a very high background level of SCE, and in the present study endoreduplicated cells with a double complement of DNA appeared under certain circumstances to have a lower SCE frequency.

The behaviour of ring chromosomes does imply a low spontaneous frequency of SCE, although it is not easy to see why breakage and SCE levels should vary among rings from different individuals, and these chromosomes may not give a valid picture of chromosome behaviour under normal circumstances. The results are also complicated by the variable amounts of ring loss, possibly due to non-disjunction of dicentric rings.

Another approach to the problem is to look at the occurrence of SCE *in vivo*. As an approximation to this, SCE frequencies in primary cell lines have been compared with those in established and virus-transformed lines (Wolff *et al.*, 1975), but there was no consistent difference

among cell types although SV40 transformed cells seemed to have a slightly raised SCE frequency. The first *in vivo* SCE study using BrdU was published by Bloom and Hsu (1975) who treated chick embryos *in ovo* with a BrdU solution dropped on to the inner shell membrane, and succeeded in obtaining sister chromatid differentiation with H 33258. They found a low SCE frequency of about 0.75 SCE per cell per two cell cycles, although the large number of microchromosomes could not be scored. About 43% of the cells had no SCEs at all, whereas such cells are never seen in *in vitro* studies. Later experiments on mice given injections or infusions of BrdU solutions also showed a low SCE frequency in marrow (Vogel and Bauknecht, 1976) and spermatogonia (Allen and Latt, 1976). In both these systems an increase in SCE frequency in response to chemical mutagens was demonstrated, using Triaziquin or Cyclophosphamide (CP), or Mitomycin C (MMC) respectively. Blood lymphocyte cultures from rabbits treated with BrdU *in vivo* have also been used to demonstrate the low background SCE level and the increase in SCE in response to MMC, ethyl methane sulphonate (EMS) and CP (Stetka and Wolff, 1976).

BrdU is itself an effective mutagen, and besides induction of chromosome alterations, it has been shown, among other effects, to cause a ten-fold increase in mutation rate to azaguanine resistance over the spontaneous rate, in human diploid fibroblasts (Stark and Littlefield, 1974) and to affect the composition of cellular RNA (Hill *et al.*, 1974) and protein (Bick and Soffer, 1976) probably

by base substitution during transcription and translation. BrdU substitution also causes the well-known sensitisation of DNA to photolysis by light of about 313 nm wavelength. Visible light causes an increase in SCE in BrdU substituted chromosomes (Ikushima and Wolff, 1974a) and the low SCE frequency in marrow cells treated *in vivo* may partly be due to their protection from light; but allowing for a maximum two-fold difference in SCE rate due to light exposure (Wolff and Perry, 1974), the *in vivo* rate is still fairly low for the concentration of BrdU that must have been incorporated to give good differentiation in staining between sister chromatids. There are still too many unknown factors to say conclusively whether SCEs do occur spontaneously, but the evidence does point to a low natural background rate.

iv. Chromosome Structure and DNA Polarity

The 2:1 ratio of singles to twins found in this work and in studies of Chinese hamster cells by Wolff and Perry (1975) indicate strongly that these chromosomes are unineme, and these restrictions on rejoining of chromatids are convincing evidence that each chromatid contains subunits of opposite polarity that represent the two polynucleotide chains of a single DNA double helix (Fig. I, 4, 3). There is no evidence from any of the observations for the occurrence of exchanges between single polynucleotide strands of the sort envisaged by Gatti and Olivieri (1973) and Gatti *et al* (1974). Such single strand exchanges would give rise to heterolabelling at M1, that is differentiation between chromatids in some regions of chromosomes that are otherwise evenly

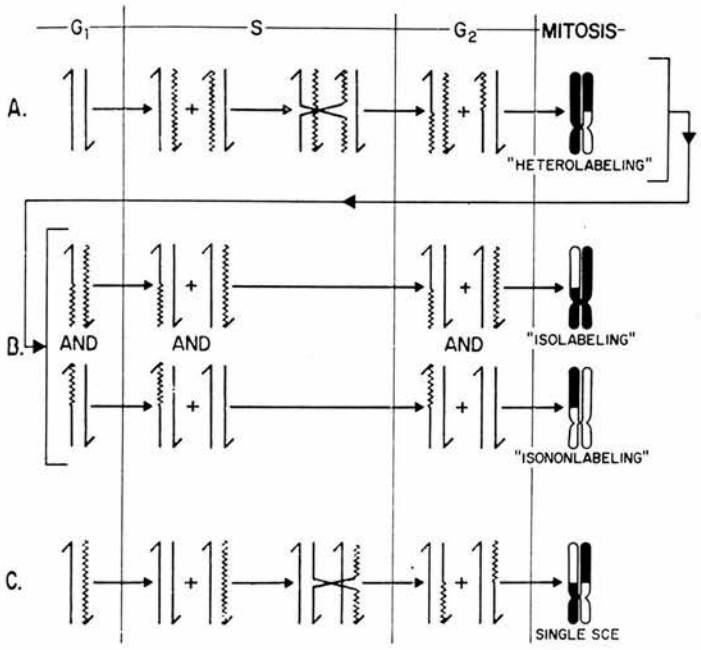
Fig. I, 4, 5.

Recombinational DNA repair and sister chromatid exchanges;

Generation of "heterolabelling" and "isolabelling"

by exchanges of single polynucleotide strands.

(from Bender et al, 1974)




Schematic diagram showing the production of exceptional label segregations and of SCE by recombinational DNA repair. The wavy lines represent labeled polynucleotide strands, while the solid areas of chromatids in the metaphase chromosomes represent labeled segments seen in autoradiographs. A, production of heterolabeling in the first post-labeling division; B, production of isolabeling and isononlabeling exceptions in the second post-labeling division (note that these are manifestations of the same event that leads to heterolabeling in the first division); C, production of single SCE in the second post-labeling division.

labelled, and isolabelling at M2 where chromatids should have different amounts of label (Fig. I, 4, 5). There is no evidence for these aberrant labelling patterns in the several thousand FPG-stained cells examined.

The issue can be complicated in systems other than short term blood cultures by incorporation of BrdU during only part of any S phase. In blood cultures the lymphocytes are thought to be in '$G_o$' at initiation of culture and the analogue is present throughout growth, but if BrdU is added to monolayer cultures the cells that are exposed may be in different stages of S phase so that cells examined at metaphase may have undergone one, two or three rounds, plus a fraction of a round of replication in the presence of BrdU. This can lead to chromosomes where, for example, late labelling regions have incorporated BrdU for three rounds and appear pale on both chromatids, or isolabelled, while the remainder of the chromatin has taken up the analogue during only the two subsequent rounds and shows normal 'harlequin' staining. This was demonstrated here in mouse A9 cells in monolayers, and also by adding BrdU to blood cultures at various times during incubation.

The specific regions of chromosomes that show asymmetry of staining after only one round of BrdU incorporation - for example, asymmetrical C-bands - would have been described as 'heterolabelled' in M1 cells, and also appear isolabelled at M2. Thus, convincing explanations for any 'isolabelling' seen are available without recourse to models of polyneme chromosomes.

Detailed information on polarity of DNA in chromosomes has been inferred from the behaviour of X-ray induced ring chromosomes (Peacock et al., 1973) where there is evidence for a limited number of switches in polarity in the DNA of the Chinese hamster genome. More sophisticated investigations using FPG technique have confirmed this (Wolff et al., 1976) and imply that there are about six such changes in polarity per genome per cycle. These are confined to the large chromosomes and do not seem to be at the centromeres, in accordance with the observations on the 'trans' arrangement of asymmetrical C-bands in centrically fused mouse chromosomes where the continuity of polarity of DNA through the centromere is clearly demonstrated. On the other hand, recent studies in Vicia suggest that polarity switches do occur in centromeric regions (Geard, 1976) of these chromosomes.

# CHAPTER FIVE

## APPLICATION OF THE FPG TECHNIQUE TO INVESTIGATION OF SCE IN RELATION TO CHROMOSOME ABERRATIONS AND DNA REPAIR

### A. INTRODUCTION

#### i. Chromosome and Chromatid Aberrations

The wide variety of chromosome aberrations induced by ionising radiation are thought to be the results of simple breakage (deletions), or breakage followed by reunion or exchange (reviewed by Evans, 1962), and have been comprehensively described and classified by several authors (see, for example, Evans, 1962; 1966; 1974; Savage, 1976). The aberrations are broadly divided into two categories according to the units of breakage and exchange: chromosome-type aberrations, where both chromatids are affected at identical loci; and chromatid-type damage, where only one chromatid of any one chromosome is involved. Exchanges may occur within (intrachanges) or between (interchanges) chromosomes or chromatids.

Chromosome-type aberrations are thought to be derived from injury to the pre-replication chromatid during $G_1$ so that the damage is duplicated along with the chromosome; while exposure to radiation of chromatids of the complete chromosome during S or $G_2$ results in damage to only one chromatid, with the exception of iso-chromatid deletions.

The transition from induction of chromosome- to chromatid-type damage occurs in late $G_1$ and early S phase (Evans and Savage, 1963; Wolff and Luippold, 1963). The possibilities for interaction of broken ends lead to a range of exchanges: complete or incomplete; asymmetrical with resultant acentric fragments; and symmetrical exchanges that do not have associated fragments unless rejoining is incomplete.

An alternative hypothesis to breakage and reunion as the mechanism for aberration formation was proposed by Revell in 1959, and according to this all aberrations were the result of a postulated exchange event (reviewed by Revell, 1974). The new FPG technique has proved valuable in aberration studies since the fate of individual chromatids is clearly visible, and it is possible to test the validity of Revell's ideas by scoring the frequency of occurrence of SCE at sites of aberration formation.

Like aberrations, SCEs were known to be induced by incorporated tritium (Brewen and Peacock, 1969a; Gibson and Prescott, 1972) or by irradiation with UV (Rommelaere et al., 1973) or X-rays (Gatti and Olivieri, 1973). With the advent of the BrdU-labelling techniques, it soon became clear not only that SCEs were induced by BrdU itself (Kato, 1974b; Wolff and Perry, 1974), but that the frequency of SCE was dramatically increased by chemical mutagens (Kato, 1974a; Latt, 1974b; Soloman and Bobrow, 1975; Perry and Evans, 1975). It was suggested that SCE might be related to a cellular repair process (Kato, 1973; 1974b; Bender et al., 1974; Wolff et al., 1974) and it is of interest to

examine the relationships of SCE to repair efficiency and to aberration formation.

ii. Mechanisms of Repair of DNA

There are now several known mechanisms for repair (reviewed by Howard-Flanders, 1973). In normal cells, altered DNA bases such as the thymine dimer, a major product of UV irradiation, can be removed by a series of enzyme steps known as excision repair (Fig. I, 5, 1), where an endonuclease makes a nick in the polynucleotide strand near the damage, leaving a 3' phosphoryl end-group that can now be recognised by an exonuclease, so that the altered nucleotides can be removed. The space is then filled in by a polymerase, and the final joining of the open ends is completed by a ligase. The first evidence for the resynthesis of DNA necessary for models of repair came from the observation of 'Unscheduled DNA Synthesis (UDS)' in irradiated nuclei that were not in S phase - i.e. not carrying out semi-conservative DNA synthesis - (Rasmussen and Painter, 1964). This radiation-stimulated synthesis was detected by auto-radiography after incorporation of isotope, and the same authors later described a more sensitive technique for detection of repair replication by equilibrium centrifugation of DNA, whereby nucleic acid that had incorporated label during repair synthesis could be separated by its altered density (Rasmussen and Painter, 1966). This was observed after ionising (Painter and Cleaver, 1967) or UV (Cleaver and Painter, 1968) radiation. A very sensitive technique for detecting areas of incorporation of label into DNA was to add BrdU, and make use of the photolysis of BrdU-substituted DNA by 313 nm light (Regan et al., 1971), subsequently

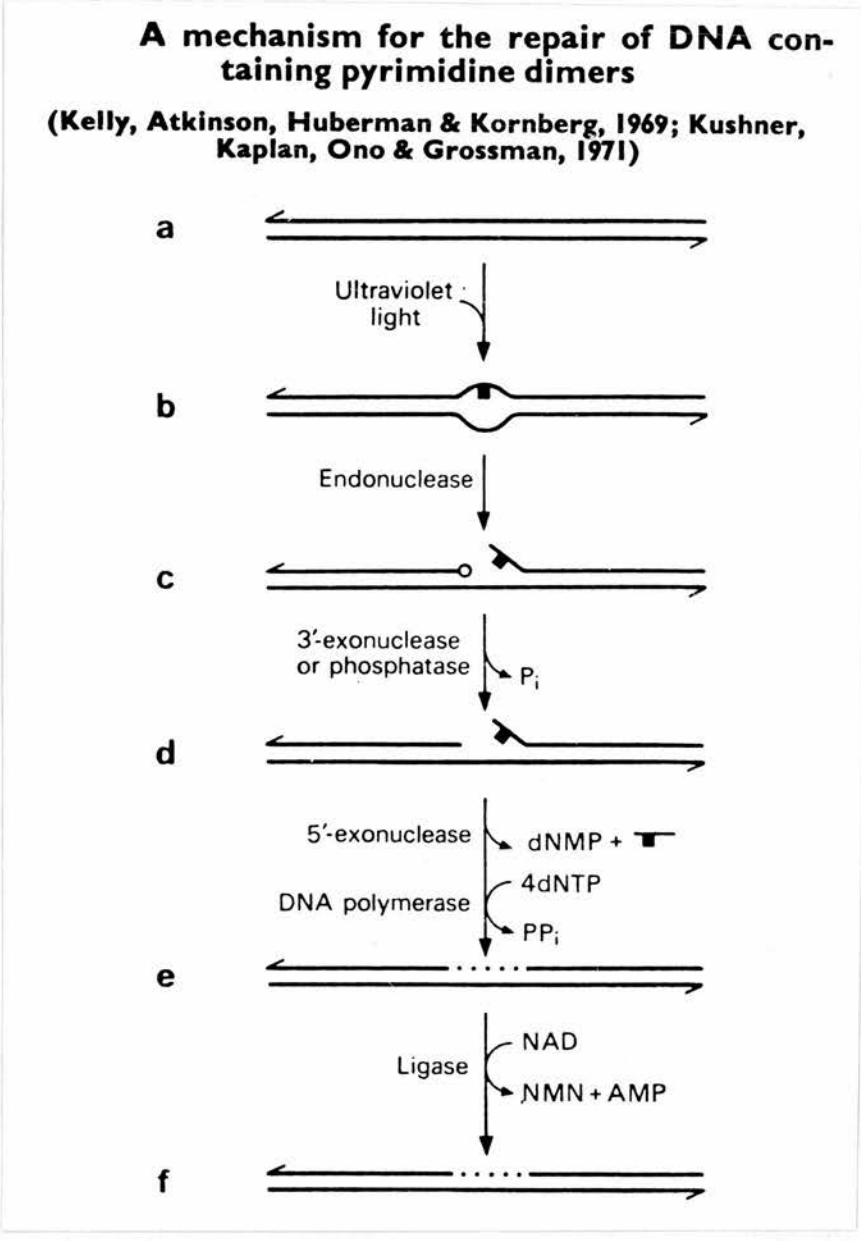



Fig. I, 5, 1. "Excision Repair."

(Diagram taken from Howard-Flanders, 1973).

a. DNA duplex.

b. Pyrimidine dimers are induced in the DNA by UV light.

c. Endonuclease cuts phosphodiester bond adjacent to the dimer leaving a 3'-phosphoryl end-group.

d. The 3'-P end-group is removed by a 3'-exonuclease, leaving a 3'-hydroxy terminus.

e. A 5'-exonuclease and DNA polymerase add nucleotides to the 3'-OH terminus, and excise the dimer along with some additional nucleotides, from the 5' end.

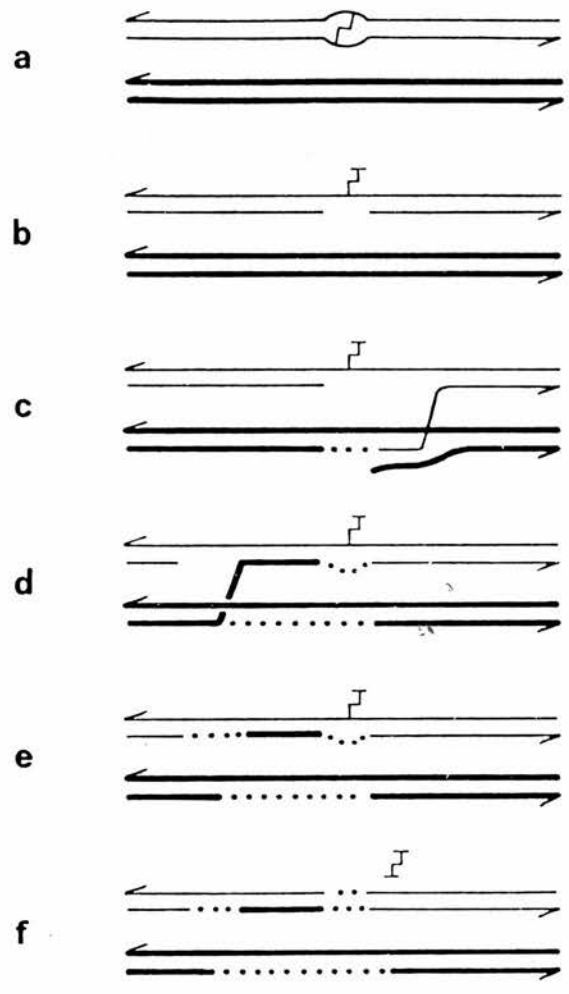
f. The repair is completed by a ligase that joins the 3'-OH and 5'-P ends.

detecting the resultant short pieces of cleaved DNA by ultracentrifugation. In prokaryotic cells and some higher animals (Regan and Cook, 1967), dimers can be directly corrected by a process known as photoreactivation, until recently thought to be absent in mammalian cells (Cleaver, 1966). There is now evidence that human cells can carry out this type of repair (Sutherland, 1974). A third repair mechanism known in mammalian cells is 'post-replication' repair (PRR) which serves to bypass damage in the DNA template and is particularly important when both DNA strands are damaged at about the same site. In prokaryotic cells this is known to be accomplished by a recombination process between the two DNA strands (Fig. I, 5, 2; and Howard-Flanders, 1973) and although PRR is known to be very efficient in mammalian cells, its mechanism is not understood. It is this type of repair that has become the focus of renewed interest following the development of the SCE detection techniques, since because of the possible involvement of recombination, a link was suspected between PRR and SCE formation (Wolff et al., 1974; Kato, 1973) [see Discussion, p.149].

There are several lines of approach in investigating the links between SCE formation and repair, and one method is to examine SCE frequency in individuals whose repair mechanisms are thought to be inefficient.

Fig. I, 5, 2.

**Proposed mechanism for recombinational repair of DNA containing an inter-strand cross-link**




a. A DNA duplex contining an interstrand crosslink, and a homologous duplex (heavy lines).

b. One strand is cut so as to free one arm of the crosslink and leave a single strand (SS) gap.

c. At the gap formed by excision of the crosslink, the strands are not immediatley rejoined because the crosslink remains attached to the SS region. Instead the strands interact with an intact portion of the homologous duplex so as to cut one strand and join in the configuration shown (repair synthesis....).

d. The gap opposite the crosslink is filled in by a change in the hydrogen bonding, as shown.

e. The strand linking the two duplexes is cut, so that both chains can be repaired.

f. With the strand opposite the crosslink repaired, the half-excised crosslink becomes again accessible to excision enzymes and is released entirely. The resulting gap is repaired.

(from Howard-Flanders, 1973.)

### iii. Effect of Age and Chromosome Constitution on Aberration Frequency and Repair Efficiency

Age-related chromosomal changes have been noted *in vivo*, where aneuploidy increases in older individuals (see Part II) and *in vitro*, where chromosome aberrations accumulate in senescent fibroblast cultures (Hayflick and Moorhead, 1961). The reduced *in vitro* lifespan of fibroblast cultures established from skin biopsies from older people (Hayflick, 1965) and the increased susceptibility to mutagenesis of cells from older individuals (Bochkov and Kuleshov, 1971) have been quoted as evidence for decreased repair efficiency in these cells. A slightly elevated frequency of exchange aberrations induced by gamma rays in cells from normal neonates compared with adults has been reported by Sasaki *et al* (1970) and increased radiosensitivity was noted in cells from individuals with abnormal chromosome constitutions (Sasaki *et al*., 1970; Higurashi and Conen, 1972), so that it was of interest to examine SCE frequency in such individuals in addition to investigating SCE levels in cells from newborn babies and elderly people.

### iv. The Chromosome Instability Syndromes and Xéroderma Pigmentosum

There are now several recognised autosomal recessive conditions involving a predisposition to developing cancer, and also a marked fragility of chromosomes in cultured cells from affected individuals. These diseases are important as they show that instability and mutation in somatic cells are associated with carcinogenesis (see Part II, Chapter One).

Bloom first described the collection of symptoms including dwarfism and sun-sensitive erythema of the face which is now known as Bloom's Syndrome (BS) in 1954, and ten years later the first report of chromosome breakage in this disease was published by German (1964b), who is now following up about 50 cases. About the same time, Schroeder published her observations on two boys with symptoms of Fanconi's Anaemia (FA), where she found that about 25% of the cells also had chromosome damage (Schroeder et al., 1964). These children have bone marrow deficiencies associated with skeletal, heart and kidney disorders, and again confirmed cases are unusual, with reports of about 100 in the literature.

Louis-Bar syndrome was described in 1941, although there had been previous clinical reports of similarly affected children (reviewed by Harnden, 1974). This disease is commonly known as Ataxia Telangiectasia (AT) and shows a wide variety of clinical abnormalities, including immunological defects, but the constant features are progressive cerebellar ataxia, or loss of muscular co-ordination and oculo-cutaneous telangiectasia due to chronic dilatation of the small blood vessels. About 250 cases are reported in the literature. There were varying reports on the cytogenetics of AT, but a great deal of evidence has accumulated recently, despite the rarity of this disease, to show that this syndrome also includes a tendency to spontaneous chromosome breakage (Hecht et al., 1966; Gropp and Flatz, 1967; Bochkov et al., 1974; Cohen et al.,

1975) although occasionally patients with no apparent chromosome aberrations are found (Pfeiffer, 1970; Schuler et al., 1972). The types of aberrations are to some extent specific to each disease, since Bloom's syndrome cells show a high frequency of chromatid interchanges, typically between homologues, resulting in triradial or quadriradial configuration (German, 1964b; German et al., 1974), and Fanconi's Anaemia cells have a high frequency of chromatid aberrations of all types. AT cells contain a wide variety of aberrations, but there are now several reports of clonal growth of lymphocytes carrying particular translocations, specifically involving chromosome 14 (Hecht et al., 1973; Bochkov et al., 1974; Cohen et al., 1975; Harnden, 1974; McCaw et al., 1975; Hook et al., 1975; Oxford et al., 1975; Hayashi and Schmid, 1975a; and my own results [Chapter Five]). This may be an important observation, since there is now some evidence for the association of specific chromosomes or translocations with certain forms of cancer, notably the Philadelphia or Ph[1] chromosome (Nowell and Hungerford, 1960) in chronic myeloid leukaemia, later shown to be in many cases a 9:22 translocation (Rowley, 1973) but also some less well-established cases (reviewed by Rowley, 1975; 1976). A 'marker' chromosome 14 (14 q+) has been reported in marrow cells and lymphoid cell lines from certain malignant lymphomas (Manolov and Manolova, 1972; Zech et al., 1976; Fukuhara et al., 1976), although other reports suggest that this may not be specific to these diseases (e.g. Fleischmann et al., 1976). The 'marker' chromosome 14 may be the very long chromosome resulting from a (14q;14q) translocation,

or may be only slightly longer than a normal 14, and in some cases this has been shown to be the result of a translocation between chromosomes 14 and 8 (Rowley, 1976; Zech *et al.*, 1976). Overall, chromosome 14 may be involved in many rearrangements, but the prominence of 14 itself is the point of interest. In AT the developing clones carrying a 'marker' are not necessarily malignant and may simply proliferate because they have some selective advantage over the other abnormal cells; in support of this there is some evidence that the clone cells have less chromosome damage (Hecht, cited by Harnden, 1974). In one of eight AT cases described by McCaw *et al* (1975, however, the proliferation of a clone of cells with the 14 q+ marker did coincide with the development of malignancy.

The chromosome instability is not confined to lymphoid cells, but has been confirmed in skin fibroblast cultures in BS and FA (German and Crippa, 1966) and in AT (Oxford *et al.*, 1975); and Cohen *et al* (1975) found that fibroblasts had in fact many more aberrations than had blood cells. This was not simply due to the necessarily longer-term culture of these cells, since normal skin cells did not show an exaggerated number of aberrations.

Apart from the clinical and cytogenetic interest, these diseases are important in attempts to understand the control of cellular DNA repair mechanisms. In the absence of convenient mammalian systems that can be manipulated in the same way as repair-defective *E. Coli* mutants, these

human chromosome instability syndromes are a rich source of information in attempts to elucidate mammalian repair systems, since these cells have increased sensitivity to various mutagens. The photosensitive disease Xeroderma Pigmentosum (XP) has been exploited in investigations of the response of human cells to UV light (Cleaver, 1968; Parrington et al., 1971) and we now know that cells from the spontaneous chromosome breakage syndromes are more sensitive to physical and chemical mutagens, and have specific sensitivities (Table I, 5, 21 ) - e.g. AT cells are very susceptible to killing and damage-induction by X- and gamma rays (see Taylor et al., 1975) and FA cells to cross-linking agents such as Mitomycin C (Sasaki and Tonomura, 1973).

Like individuals with the chromosome instability syndromes, XP patients have a high cancer risk, although here the disease is much more specific and often results in skin cancer. The study of the link between UV-susceptibility and cancer has led to the discovery (Cleaver, 1969a; Setlow et al., 1969) of the particular enzyme defect in XP where the cells are unable to remove from their DNA the products of UV irradiation, of which the most abundant and easiest to study is the thymine-dimer. XP cells specifically lack an active endonuclease needed for the first incision step (Cleaver, 1969a) and consequently these UV-sensitive cells are not abnormally sensitive to X-rays and gamma rays which produce single stranded (SS) breaks directly, so that the incision step is not necessary before

repair can take place. The situation in XP may be more complex than was suspected, as there is now evidence that normal human cells do have a photoreactivation system (Sutherland, 1974), previously thought to be absent in mammalian cells; and that the effectiveness of this photoreactivation repair is reduced in XP cells (Sutherland et al., 1975). XP is genetically heterogeneous with at least five complementation groups now known, and designated groups A to E (de Weerd-Kastelein et al., 1972). There is also a sixth 'variant' type of XP where although UV repair is reduced (Day, 1975) excision repair is normal; but here post-replication repair (PRR) is inefficient (Lehmann et al., 1975) and photoreactivation is reduced (Sutherland and Oliver, 1975).

## v. Caffeine: Effects on Repair

Besides looking at SCE in association with spontaneous chromosome damage, we can investigate the effect of radiation and chemical mutagens on both normal and repair-deficient cells. A further approach used widely in experiments with repair in bacterial systems, is to interfere with the repair mechanisms by adding certain chemicals.

Caffeine (a methylated oxypurine) is known to sensitise bacteria to UV so as to give increased cell-killing and mutation rate (Witkin, 1969 a;b). This effect was attributed to a caffeine-mediated inhibition of excision of pyrimidine dimers that had been induced by UV irradiation (Setlow and Carrier, 1968). It was later shown that

caffeine post-treatments potentiated the UV induction of chromosome aberrations and mutations in several plant and mammalian systems (Kihlman et al., 1973), but since in mammalian cells thymine dimer excision (Regan et al., 1968) and unscheduled DNA synthesis (Cleaver, 1969b) were not reduced by caffeine treatment, in contrast to the effects in bacteria, it was clear that some process other than excision repair was being inhibited. It was suggested that the caffeine-sensitive mechanism was post-replication repair (PRR), and this seemed to be inhibited in mouse L cells where caffeine potentiates the effects of MMC (Rauth et al., 1970) and UV irradiation (Rauth, 1967). On the other hand, caffeine did not increase the sensitivity to UV of HeLa cells, even in a cell line known to be abnormally sensitive to MMS and to UV radiation (Wilkinson et al., 1970) or of a Chinese hamster line (Arlett, 1967); and PRR in response to sulphur mustard or to MNU was reported to be unaffected by caffeine in HeLa cells (Roberts and Ward, 1973). It thus became clear that various cell lines have a range of responses to caffeine, and vary in their capacity to carry out the postulated caffeine-sensitive step in PRR.

In Chinese hamster cells, non-toxic concentrations of caffeine can lower the UV-induced mutation frequency (Trosko and Chu, 1973); but Roberts and Sturrock (1973) found a completely different effect using N-methyl N-nitrosourea, where caffeine post-treatment increased the mutation frequency. This may reflect differences in the repair

mechanism recruited for the different types of damage following these two types of insult to the cells, and the effects of caffeine in each appears to lead to different consequences.

If caffeine affects repair, it may also affect chromosome aberration production; and Kato (1974a) has shown that in Chinese hamster cells where UV produces an increase in both SCE and chromosome aberrations, caffeine post-treatment does potentiate the aberration effects, but depresses the numbers of UV-induced SCEs, so that the relationships are not straightforward. However, since caffeine has effects on many aspects of cell metabolism (reviewed by Trosko and Chu, 1975) such as inhibiting AMP phosphodiesterase and affecting membrane permeability, we may be seeing not a direct action on repair processes but a more general effect. Lehmann (cited by Trosko and Chu, 1975) has found synergism between caffeine and mutagens in human Xeroderma Pigmentosum (XP) variant cells, known to have reduced post-replication repair, and this is further evidence that caffeine effects are involved somehow with DNA repair in human cells. In human lymphocytes treated with caffeine at the same time as they were exposed to mutagens, a synergistic effect on aberration induction was noted by Brøgger (1974), so that it was also interesting to examine SCE frequency in a similar system in the present study.

The following experiments were designed to investigate SCE in relation to age, chromosome constitution, repair efficiency, and radiation or chemical mutagenesis.

## B. SCE FREQUENCIES AT DIFFERENT AGES AND IN CHROMOSOMALLY ABNORMAL INDIVIDUALS

### i. SCE in the Very Young and Very Old

The frequency of SCE for 12 newborn babies (six females and six males) is shown in Table I, 5, 1 along with the average SCE levels for adults aged 20-30 years. The results show very little variation and the only unusual SCE scores are in two male babies who had 20.1 and 22.1 SCE per cell (or 0.451 and 0.48 per chromosome) at 160 μM (50 μg/ml) BrdU (see Appendix II). In 't test' comparisons between groups of male and female adults and babies (Appendix II), the male babies of group IV had a significantly higher SCE frequency than all the other three groups (Appendix II). Table I, 5, 2 shows the SCE frequencies for seven individuals aged 71-89 years. The numbers of cells with chromosome-type and chromatid-type aberrations (B + C cells) in three-day cultures are also shown because this may be related to the efficiency of repair mechanisms, and it was thought that SCE formation might also be in some way related to repair processes. The average SCE score for the three individuals with 2% (B + C) cells is 11 per cell, while the mean for the four old people with higher chromosome aberration scores is 9.7; this is not a dramatic difference and the numbers are small, but it may indicate a trend to a reduction in SCE in cells with increased aberration levels. However, it is much more probable that the difference can be accounted for by normal variation among cultures.

TABLE I 5 1.

Frequency of SCE in newborn babies and in adults.

| Conc. BrdU (μM) | 10 | | 25 | | 160 | |
|---|---|---|---|---|---|---|
| | No. of cells | SCE/ cell | No. of cells | SCE/ cell | No. of cells | SCE/ cell |
| Newborn | 21 | 7.2 | 11 | 7.8 | 28 | 22.1 |
| | | | 22 | 9.8 | 8 | 16.8 |
| | | | 33 | 11.1 | 7 | 20.1 |
| | | | 50 | 9.9 | 8 | 14.8 |
| | | | 25 | 8.7 | 7 | 12.4 |
| | | | | | 8 | 14.6 |
| Total | 21 | 7.2 | 141 | 9.8 | 66 | 14.0 |
| Adults (20-30 yrs) | 26[a] | 7.2 | 499[b] | 9.6 | 98[c] | 12.5 |

TABLE I 5 2.

Frequency of SCE and Aberrations in old people.

| | Age | SCE/Cell[d] | B+C Cells as % Total |
|---|---|---|---|
| Old People. | 72 | 11.2 | 2 |
| | 76 | 7.8 | 6 |
| | 75 | 11.9 | 4 |
| | 80 | 10.9 | 2 |
| | 81 | 10.9 | 2 |
| | 71 | 9.2 | 5 |
| | 89 | 9.9 | 4 |
| Total | | 10.1 | 3.6 |

a. 1 individual b. 14 individuals c. 7 individuals
d. Average from 20-30 cells (25μM BrdU)
e. Chromosome (C) and Chromatid (B) aberrations; average from 50-100 cells from 72hour cultures.

TABLE I 5 3.

SCE frequency in an abnormal E group chromosome compared with the normal E group.

| | | Abnormal[a] | E group[b] |
|---|---|---|---|
| Total SCE | | 629 | 557 |
| Expected Proportion | % No. | 1.5 9.4 | 4.71 26 |
| Observed Proportion | % No. | 1.75 11 | 4.71 26 |

a. SCE in cells where abnormal chromosome identified

b. SCE in cells where all three normal E group chromosomes identified.

ii. SCE Frequencies in Individuals with Constitutional Chromosome Abnormalities

a. Abnormal chromosome 18. An individual was examined who had the karyotype 46XX,t (18; acrocentric) where the abnormal chromosome has the short arms of an acrocentric chromosome and most of the long arms of chromosome 18. The total SCE per cell were scored, and the frequency of SCE in the abnormal chromosome and in the normal E group chromosomes noted (Table I, 5, 3). In 69 cells the overall SCE frequency was normal (10.4 per cell). In the cells where the abnormal chromosome was identified there was a total of 629 SCEs, 11 of which were in the abnormal chromosomes. Since SCEs occur with a frequency proportional to the metaphase length of a chromosome (see Chapter Three), about 1.5% of the total SCEs would be expected to occur in this chromosome - i.e. 9.4 SCEs, a figure quite close to the observed number of 11. The expected number of SCEs in the other three E group chromosomes with a total length of 4.71%, is 26, exactly the number observed.

b. Down's Syndrome.

1. Trisomy 21. Because abnormalities of radiosensitivity and of repair synthesis have been reported in individuals with trisomy 21, I have examined SCE frequency in 11 Down's syndrome patients and 10 controls from the same institution. The patients were aged 22-58 years except for one control aged 81 who had an average SCE score. The numbers of SCEs in the G-group chromosomes were also compared. The results are shown in Table I, 5, 4a. Since there is no difference

TABLE I 5 4.

a. SCE in patients with Trisomy 21

and in controls from the same institution.

| | SCE/chromosome (25μM BrdU) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Males | | | Females | | | Total | | |
| | Chromo-somes | SCE | SCE/ chrm | Chromo-somes | SCE | SCE/ chrm | Chromo-somes | SCE | SCE/ chrm |
| Trisomy 21 | 2752 | 716 | 0.26 | 9144 | 2532 | 0.28 | 11896 | 3248 | 0.27 |
| Controls | 3703 | 976 | 0.26 | 9745 | 2701 | 0.28 | 13488 | 3677 | 0.27 |

TABLE I 5 4.

B. SCE frequency in G group chromosomes .

| | Trisomy 21 | | Controls | |
|---|---|---|---|---|
| | Males | Females | Males | Females |
| Total G+Y chromosomes | 239 | 873 | 322 | 759 |
| Expected | 45.8 | 111 | 53.7 | 83.5 |
| Observed | 32 | 77 | 28 | 66 |
| $Chi^2$ | 4.16 | 10.48 | 12.69 | 3.54 |
| P | $0.02 < p < 0.05$ | $p < 0.01$ | $p < 0.001$ | $p < 0.05$ |

in the overall frequency of SCE per chromosome between trisomy 21 patients and controls, no effect of the extra G-group chromosome was expected. The observed frequencies of SCE per G-group chromosome, along with the numbers expected according to length, are shown in Table I, 5, 4b. There are fewer than expected SCEs in these chromosomes but this is a normal finding (see Chapter Three) probably due to scoring difficulties. The frequency of SCEs in the G-group chromosomes is not high in the trisomic cells, although the under-representation of SCE in G chromosomes is more marked in male controls. The <u>total</u> numbers of G-group chromosomes were used for comparison of normal and trisomic cells to avoid complications due to the extra G-group member in Down's syndrome.

2. <u>Patient with karyotype 46, XY, t, (14q; 21q)</u>. An additional patient with trisomy 21 was examined because he had a Robertsonian translocation between chromosomes 14 and 21. Once again SCEs were scored noting their presence in the translocation chromosome. The overall SCE frequency for this individual, 7.8 per cell in 83 cells, is within the normal range at 25 μM BrdU. Assuming the length of this chromosome (estimated at slightly less than the total lengths of chromosomes 14 + 21, and taken as a proportion of a <u>47 + 21</u> cell) is about 2.65% of the genome, the expected number of SCEs in this chromosome, out of a total of 440 SCE in the cells where the abnormal chromosome was identified, is 11.7. The observed number of 16 is slightly higher than expected, especially as 21s usually have a lower

frequency than expected from their length. However, there was some difficulty in distinguishing this chromosome from those of the C-group in the FPG stained cells, and this may have raised artificially the number of SCEs assigned to the translocation chromosome.

In summary, it is clear that age and chromosome constitution have no marked effect on SCE frequency, except that some babies tend to have slightly higher than average SCE frequencies. The incidence of SCE in the two abnormal translocation chromosomes examined is also normal.

## C. SPONTANEOUS ABERRATIONS: THE CHROMOSOME INSTABILITY SYNDROMES

The possibility that SCE events may be in some way related to 'repair synthesis', and the relationship between SCE and aberration formation, can be investigated by examination of cells from the chromosome breakage syndromes. The high spontaneous levels of chromosome damage in affected individuals point to deficiencies in repair and the strong tendency to develop cancers may be associated with the same defects.

### i. Ataxia Telangiectasia (AT)

The first investigation involved two brothers with this disease (Appendix II). I was later able to investigate their unaffected sib, probably a heterozygote and their mother, an obligate heterozygote for the autosomal recessive gene implicated in this condition. The older brother, DB, 11 years old when his blood was first cultured, was the more severely affected physically and had a cleft lip and palate, while his younger sib, SB, at 7 years old had a greatly reduced blood IgA level, a common feature of AT.

#### a. Chromosome aberrations in AT patients

1. Frequency of aberrations. Orcein analysis of the first cultures revealed fairly frequent endoreduplicated and pulverised cells, and a total of about 15% of the cells had chromosome and chromatid aberrations. The aberrations were classified according to the notation of Buckton et al (1962a)

TABLE I 5 5

Chromosome and Chromatid Aberrations in blood cells from two male Ataxia Telangiectasia patients and their mother and brother

i. DB (d.o.b. 25/3/63)

| Date | Culture length (days) | Abnormal Cells | | | | Total cells | % B+Cu+Cs |
|---|---|---|---|---|---|---|---|
| | | B | Cu | Cs | Total | | |
| 6/74* | 2[a] | 1 | 1 | 1 | 3 | 50 | 6 |
| | 3[a] | 6 | 2 | 3 | 11 | 50 | 22 |
| 9/74 | 2 | 1 | 1 | 6 | 8 | 31 | 26 |
| | 3 | 3 | 1 | 7 | 11 | 33 | 33 |
| 12/75 | 2[b] | 4 | 6 | 4 | 14 | 35 | 40 |
| | 3 | 2 | 2 | 8 | 12 | 45 | 27 |
| 6/76 | 2[b] | 2 | 4 | 8 | 14 | 30 | 47 |
| | 3[b] | 1 | 0 | 2 | 3 | 30 | 10 |
| Total | 2+3 | 20 | 17 | 39 | 76 | 304 | 25 |

ii. SB (d.o.b. 24/6/76)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 6/74* | 2[a] | 0 | 3 | 4 | 7 | 30 | 23 |
| | 3[a] | 3 | 2 | 0 | 5 | 50 | 10 |
| 9/74 | 2 | 1 | 3 | 7 | 11 | 25 | 44 |
| | 3 | 1 | 0 | 4 | 5 | 15 | 33 |
| 12/75 | 3 | 2 | 1 | 7 | 10 | 40 | 25 |
| 6/76 | 2[b] | 0 | 2 | 6 | 8 | 18 | 44 |
| | 3[b] | 4 | 2 | 10 | 16 | 40 | 40 |
| Total | 2+3 | 11 | 13 | 38 | 62 | 218 | 28 |

iii. normal sib WB (d.o.b. 17/11/60)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 9/74 | 2+3[b] | 2 | 0 | 0 | 2 | 75 | 2.7 |

iv. mother (d.o.b. 22/4/40)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 9/74 | 2+3[b] | 2 | 0 | 0 | 2 | 60 | 3.3 |

Analysis on G banded cells except where otherwise stated.
a. Orcein analysis only. b. Orcein and ASG results pooled.
* Results of Miss M.L. O'Riordan.

where cells with chromatid-type aberrations only are classed as 'B' cells, while those with chromosome-type aberrations are called 'C' cells and divided into 'Cs' cells with stable abnormalities such as translocations, and 'Cu' cells with unstable aberrations, such as dicentrics, rings and acentric fragments. Total numbers of aberrations were also calculated as 'C' cells may contain both chromosome- and chromatid-type damage. The results of analyses on four separate occasions are shown in Tables I, 5, 5 (i) and (ii). These Tables illustrate the high frequency of both stable and unstable aberrations even without the inclusion of chromatid aberrations. Chromatid gaps were more frequent in three-day cultures from both brothers but culture length did not have a consistent effect on the total proportions of abnormal cells, as these were more frequent in the 'early' culture for SB but in 'late' culture for DB on the first analysis. There have been reports of above-average numbers of chromosome abnormalities in AT heterozygotes, but in the mother and unaffected brother of these two patients there was no evidence for chromosome instability, the only damage seen being two chromatid gaps in 30 cells from each relative (Table I, 5, 5iii).

A second, apparently unrelated family with AT, was also found in Edinburgh. Two affected sisters (KS and FS) were examined and the cultures shown to have a generally better mitotic index than in the first two patients studied and a slightly lower incidence of chromosome breakage (Table I, 5, 6) particularly in the older sib.

TABLE I 5 6.

Chromosome and Chromatid Aberrations in Blood from two sisters with Ataxia Telangiectasia.

| Patient | Cult. length (days) | Abnormal Cells* | | | | Total cells | % B+Cu+Cs |
|---|---|---|---|---|---|---|---|
| | | B | Cu | Cs | Total | | |
| KS (14yr) | 2 | 1 | 1 | 3 | 5 | 30 | 17 |
| | 3 | 3 | | 1 | 4 | 30 | 13 |
| FS (10yr) | 2 | 2 | | 4 | 6 | 30 | 20 |
| | 3 | 1 | | 5 | 6 | 30 | 20 |

* results pooled from orcein and ASG analysis.

2. Chromosomes involved. The chromosome rearrangements detected in G-banded cells were examined for evidence of a preferential involvement of certain chromosomes expecially chromosome 14, a chromosome that has been reported to be specifically involved in aberrations in cells from AT patients (see p.107). The numbers of cells analysed in this way are shown below, and the complete data are presented in Table I, 5, 7.

| Patient | Total cells (ASG) | Total cells (Cu + Cs) |
|---|---|---|
| DB | 180 | 39 |
| SB | 128 | 32 |
| KS | 40 | 8 |
| FS | 40 | 4 |

If we assume that each chromosome should be involved in aberrations with a frequency proportional to its metaphase length, a comparison of observed frequencies of involvement with expected frequencies according to length shows a pattern that is clearly non-random (Table I, 5, 7). In the total data chromosome 14 is commonly involved in aberrations, in addition to chromosome 17, but chromosome 7 has a greater deviation from expected values than either of these. The X chromosome is also involved more often than expected and this is very striking in both male and female cells (Table I, 5, 7). This is interesting in the light of X-ray studies that showed the X chromosome was rarely implicated (e.g. Seabright, 1973b), although more recent

analyses have not confirmed that the X is spared involvement in rearrangements (Savage et al., 1973; Buckton, 1976). There is no evidence in these AT studies for preferential breakage of regions at or near the centromeres such as those so frequently involved in interchanges in cells from BS patients (German et al., 1974). The breaks in chromosome 14 in the present study are apparently in bands 14 q 11-3, in accordance with other reported observations (see p. 107 and below).

The involvement of chromosome 7 is most noticeable in FS, but is also significant in the two male patients, while chromosome 14 is most frequently implicated in DB. Apparently identical translocations between chromosomes 7 and 14 were seen in FS (in one cell out of 40) and in SB (on two separate occasions; in 1/15 and 1/30 cells). A different t (7; 14) was also found in one out of 33 of DB's cells. In this patient chromosome 14 was also involved in translocations with chromosomes 14, 5 and 9 and in a chromatid interchange with chromosome 1 and a dicentric with 7. Pericentric inversions of chromosome 7 (inv. [7] [p14-5q36]) were seen in three of the four patients, occurring in 2/40 cells (FS), 2/15 and 2/40 cells of two separate cultures from SB and 1/30 and 1/31 cells from two cultures from DB. All but one of these occurred in three-day cultures so may not represent an in vivo clone but nevertheless indicate an abnormal propensity of chromosome 7 to undergo inversions in cells from AT.

TABLE I 5 7

Chromosomes involved in rearrangements in Ataxia Telangiectasia

| Chromosome | No. of times involved | | | | | No. of times involved:- expected | | $Chi^2$ |
|---|---|---|---|---|---|---|---|---|
| | males | | females | | Total | | | |
| | DB | SB | KS | FS | | % | No. | |
| 1 | 2 | 2 | | 1 | 5 | 8.44 | 12.2 | 4.3* |
| 2 | 4 | 3 | | 1 | 7 | 8.02 | 11.6 | 1.82 |
| 3 | 4 | 1 | | | 5 | 6.83 | 9.9 | 2.43 |
| 4 | 4 | 3 | | | 7 | 6.3 | 9.14 | 0.05 |
| 5 | 2 | 3 | | | 5 | 6.08 | 8.82 | 1.25 |
| 6 | 3 | 4 | | | 7 | 5.9 | 8.56 | 0.28 |
| 7 | 5 | 9 | | 3 | 17 | 5.36 | 7.77 | 10.96*** |
| 8 | 1 | 4 | 1 | | 6 | 4.93 | 7.15 | 0.18 |
| 9 | 2 | 0 | 1 | | 3 | 4.8 | 6.96 | 2.25 |
| 10 | 4 | 2 | 1 | 1 | 8 | 4.59 | 6.66 | 0.27 |
| 11 | 2 | 1 | | 1 | 4 | 4.61 | 6.68 | 1.08 |
| 12 | 2 | 4 | | | 6 | 4.66 | 6.76 | 0.09 |
| X | | | | 4 | 4 | 5.12 | 1.08 | 7.89** |
| (female) X (male) | 3 | 5 | | | 8 | 2.56 | 3.17 | 7.36** |
| 13 | | 2 | | | 2 | 3.74 | 5.42 | 2.16 |
| 14 | 7 | 3 | 1 | 1 | 12 | 3.56 | 5.16 | 9.07** |
| 15 | 3 | | | | 3 | 3.46 | 5.02 | 0.81 |
| 16 | 3 | 2 | | | 5 | 3.36 | 4.87 | 0.003 |
| 17 | 4 | 5 | 1 | 1 | 11 | 3.25 | 4.7 | 8.4** |
| 18 | 1 | | | 1 | 2 | 2.93 | 4.25 | 1.19 |
| 19 | 2 | 2 | | 1 | 5 | 2.67 | 3.87 | 0.33 |
| 20 | 5 | 1 | | | 6 | 2.56 | 3.7 | 1.43 |
| 21 | 1 | 1 | | 1 | 3 | 1.9 | 2.76 | 0.02 |
| 22 | 2 | | | | 2 | 2.04 | 2.96 | 0.31 |
| Y | 1 | | | | 1 | 2.15 | 2.67 | 1.04 |
| Total | 67 | 57 | 5 | 16 | 145 | | | |

Chromosomes underlined are involved more often than expected, the excess being significant by the chi squared test.

*p < 0.05; **p < 0.01; ***p < 0.001

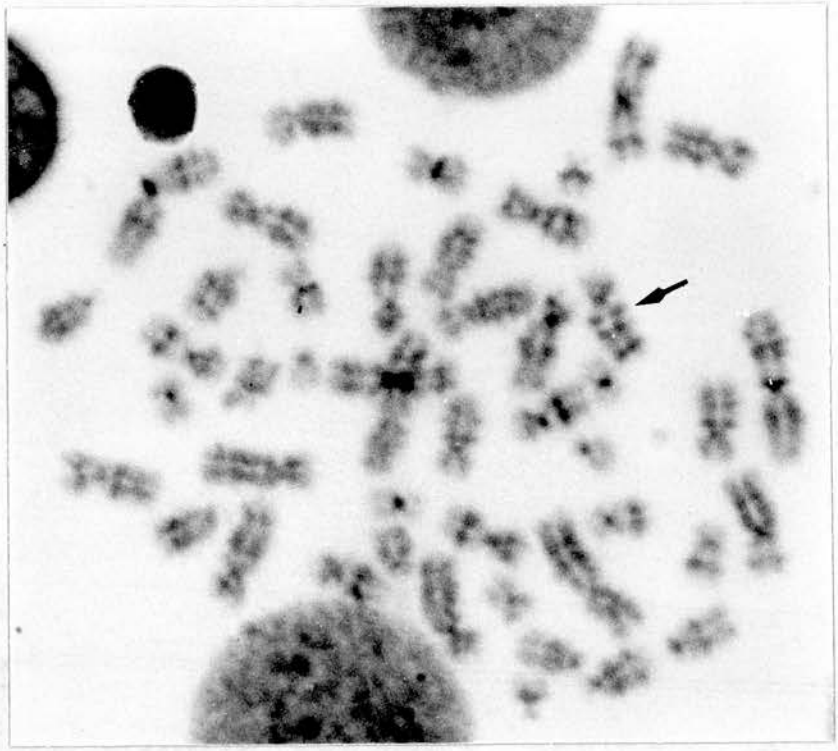
Fig. I, 5, 3a.

G-banded lymphocyte from a patient with Ataxia Telangiectasia, showing a pericentric inversion of chromosome 7. inv. (7) (p15;q33-4)

Fig. I, 5, 3b.

G-banded AT cell, showing a translocation between two chromosomes number 14.

t(14;14) (q11;q32)



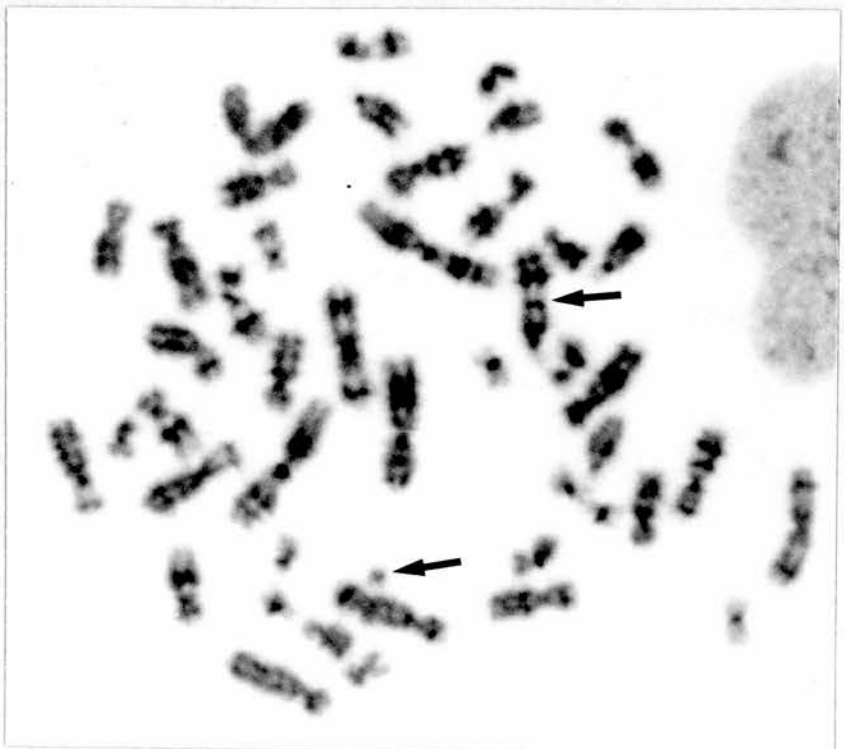


Fig. I, 5, 3c.

G-banded cell from a patient with Ataxia Telangiectasia, showing a rearrangement where most of chromosome 14 is translocated on to the long arms of chromosome 7.
t(7q;14p) (q11; p11).

Fig. I, 5, 3d.

G-banded AT cell showing a different rearrangement involving chromosomes 14 and 7.
t(7q;14q) (q32-5;q13)

There have been reports of the specific involvement of chromosomes 7 and 14 in translocations in lymphocytes from individuals other than AT patients (Beatty-de Sana et al., 1975; Hecht et al., 1975; Welch and Lee, 1975) with frequent implication of similar break-points (particularly 14q12), yet these chromosomes are not preferentially affected by X-irradiation (San Roman and Bobrow, 1973; Seabright, 1973a; Buckton, 1976), suggesting that a simple explanation such as close proximity of 7p and 14q at interphase is inadequate. Selection for these cells is a strong possibility and their occurrence may simply reflect an ability to survive in cultures; but the association of clones carrying cytogenetic markers involving chromosomes 7 and 14 in AT with the development of leukaemia leads to the tentative proposal that the appearance of such rearrangements reveals an early step in the process of carcinogenesis (Hecht et al., 1975).

b. Sister Chromatid Exchanges in Ataxia Telangiectasia Patients. Blood cultures from the two brothers DB and SB were treated with 50 μg per ml (160 μM) BrdU throughout three-day culture periods, and SCEs were scored on slides stained by the FPG technique. The results, along with scores from adult and baby controls, are presented in Appendix II. SCE levels in AT cells did not differ from normal SCE frequencies. When blood was cultured at a later date, the results using 25 μM BrdU also showed a fairly normal level, but in one sib (DB) the average score of six per cell was low compared with his brother's SCE frequency of 10 per cell (Table I, 5, 8), and on re-examining the data from Appendix II it was interesting to note the slightly

TABLE I 5 8.

Frequency of SCE in AT patients and controls.

| Culture | Conc. BrdU (μM) | Patient | Ave.SCE/ cell | cells |
|---|---|---|---|---|
| 1 | 160 | DB | 11.2 | 25 |
| | | SB | 13.2 | 25 |
| | | control | 12.1 | 58 |
| 2 | 25 | DB | 6 | 37 |
| | | SB | 10 | 42 |
| | | control | 10 | 52 |
| 3 | 25 | DB | 7.6 | 26 |
| | | SB | 4.8 | 3.5 |
| | | KS | 7.9 | 20 |
| | | FS | 8 | 21 |
| | | control | 9.6 | 25 |

1, 2 and 3;- results from three separate culture occasions.

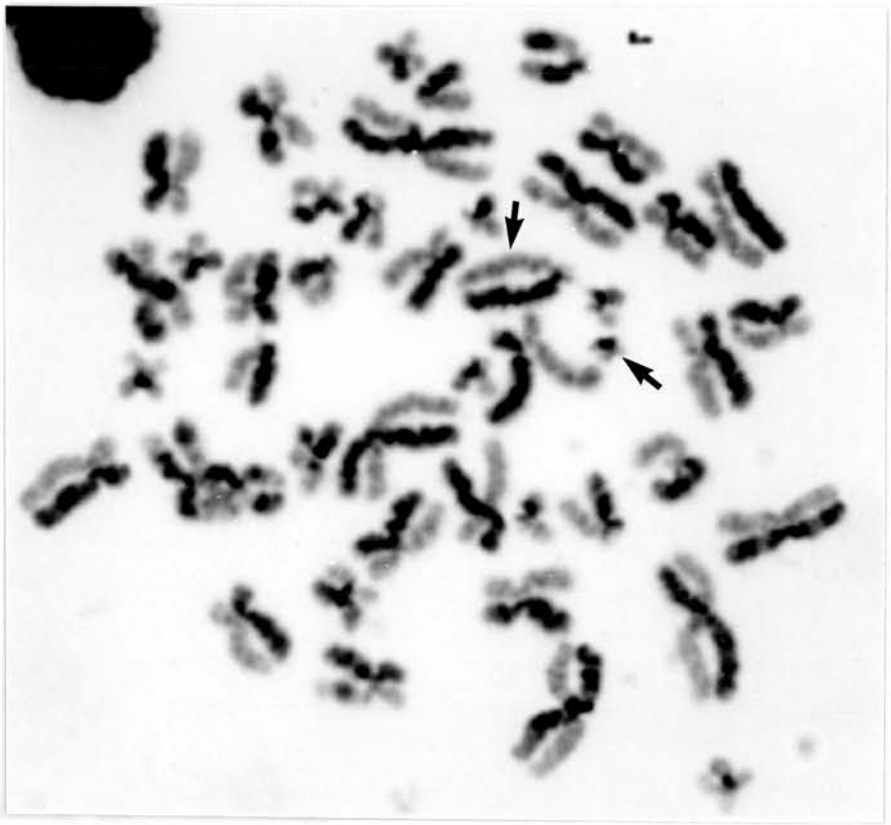


Fig. I, 5, 4.

"Harlequin"stained cell from a patient with Ataxia Telangiectasia, showing a typical spontaneously occurring translocation between two D-group chromosomes (probably t(14;14), arrows). This cell also has 9 SCEs, so that the frequency of these events is entirely normal, despite the high level of spontaneous chromosome damage in lymphocytes from patients with this disease.

lower level of 11.2 in cells from the same patient compared with 13.2 in cells from his brother (using 160 μM BrdU). These differences are very slight and are within the range of culture variations, but later scores for the two boys and also for the sisters KS and FS indicate a consistent, although slight, depression in SCE frequency in cells from AT patients.

ii. Fanconi's Anaemia

a. Aberrations in FA cells. Routine blood cultures from a seven-year old girl with FA and from her mother showed a high spontaneous rate of damage, expecially of the chromatid type, in the Fanconi's Anaemia patient, and a much less marked increase in her mother (Table I, 5, 9), while 20 cells from the father, examined after each of two- or three-day cultures, had one chromatid gap each, a not uncommon finding among normal individuals (Results of Mrs. G. Hamilton).

b. SCE in FA cells. Despite this high level of chromatid damage in cells from the individual with FA, cells from this patient had normal SCE frequency at 25 μM BrdU (Table I, 5, 9).

TABLE I 5 9.

SCE and chromosome aberrations in Fanconi's Anaemia.

| Subject | 2 day culture | | | 3 day culture | | | | Ave SCE/ cell | cells |
|---|---|---|---|---|---|---|---|---|---|
| | B cells | Cu cells | Total scored | B cells | Cu cells | Cs cells | Total scored | | |
| Fanconi's Anaemia patient | 16 | 2 | 30 | 10 | 2 | 2 | 30 | 11.8 | 52 |
| Mother | 6 | | 20 | 1 | | | 20 | 9.2 | 25 |
| Control | | | | | | | | 10.2 | 23 |

## D. X-RAY INDUCED DAMAGE

### i. X-Rays in vivo: Patients treated with X-Rays for Ankylosing Spondylitis

Nine males aged from 45 to 68 years who had been given exposures of 1500-2500 rad of X-rays to the spine 11 to 28 years previously were tested for SCE frequency. The results in Table I, 5, 10 show that there is still a significant amount of chromosome damage seen in two-day blood cultures (results of K. Buckton and G. Hamilton), but the SCE results do not show a dramatic difference from control frequencies of about 12-14 SCE per cell at 160 μM BrdU and 10-12 SCE per cell at 50 μM BrdU. However, the rather low rate of SCE (seven per cell) in SP4 and the high frequency in SP5 (15.5 at 50 μM and 20 at 160 μM BrdU) are interesting.

An interesting clone of cells was present in a culture from one of these patients, where chromosome 17 carried clearly visible satellites on the short arms in the presence of an apparently normal complete set of acrocentric chromosomes. This aberrant chromosome occurred in more than half of the cells in the culture, and was not found in a parallel culture grown (using F10 medium without BrdU) for routine chromosome analysis by G-banding. This situation is probably a good example of selection by culture conditions, and it may also be important that the abnormal chromosome was observed in M2 cells (i.e. cells in their second *in vitro* metaphase) after treatment with BrdU, whereas the cells examined using G-banding were from a 48 hour culture where the majority were presumably M1 cells and there was no sign of this

TABLE I 5 10

Patients treated with X-rays for Ankylosing Spondylitis

| Patient | Years since X-rays | total dose (rad) | Abnormal Cells (48h)[a] | | | Total Cells Scored | 160 µM BrdU | | 50 µM BrdU | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | B | Cu | Cs | | Cells | Ave. SCE/ cell | Cells | Ave. SCE/ cell |
| SP4 | 11 | 1500 | 1 | 1 | 6 | 30 | - | - | 25 | 7 |
| SP5 | 17 | 2000 | 0 | 0 | 4 | 50 | 28 | 20 | 29 | 15.5 |
| SP6 | 20 | 1800 | 2 | 0 | 4 | 50 | 28 | 14 | 28 | 11 |
| SP3 | 23 | 2000 | 0 | 1 | 3 | 30 | 25 | 14 | 30 | 11 |
| SP1 | 24 | 2000 | 0 | 2 | 0 | 30 | 29 | 11.5 | 30 | 8.5 |
| SP11 | 24 | ? | | not done | | | - | - | 30 | 11 |
| SP7 | 28 | 2500 | 1 | 0 | 9 | 50 | - | - | 29 | 10 |
| SP2 | ? | ? | | not done | | | - | - | 5 | 12 |
| SP8 | ? | ? | 0 | 0 | 8 | 50 | - | - | 31 | 13 |

a. Analysis carried out by K.E. Buckton and G. Hamilton

particular clone.

ii. X-Rays In Vitro: Normal Cells

a. SCE frequencies after X-irradiation. Whole blood cultures containing 160 μM BrdU were irradiated with doses of 100-1000 rad X-rays at varying times during incubation. The results were not very satisfactory as mitotic indices were very low, and no SCE scores were obtained from cultures given more than 200 rad. This may be partly due to the sensitisation to DNA damage and cell killing by X-rays, caused by BrdU incorporation (Dewey and Humphrey, 1965). The results for three separate experiments, Exp. 1, Exp. 3 and Exp. 6, are shown in Table I, 5, 11. Blood was irradiated in $G_0$ by exposing the whole culture, after addition of blood to the medium, immediately before incubation. To try to expose cells in their second S phase in vitro ($S_2$), or in $G_2$, irradiation was carried out 11-12 or two to three hours before fixation, respectively. These were pilot experiments, so that I did not use tritiated thymidine pulses at the time of irradiation to check the stage of the cell cycle of cells analysed.

The results (Table I, 5, 11) made it clear that these doses of X-rays were not having a dramatic effect on SCE frequency despite the high proportion of abnormal cells produced (about 30% in 48-hour cultures after 200 r given in $G_0$; Buckton, 1976), so that no further experiments of this sort were carried out. The results show a very slight tendency to an increased SCE frequency after irradiation

TABLE I 5 11

Effect of X-rays on SCE frequency in vitro

i. 25 μM BrdU

| Cell cycle stage | X-ray dose (r) | Exp 3 | | Exp 6 | |
|---|---|---|---|---|---|
| | | Cells scored | Ave. SCE/ cell | Cells scored | Ave. SCE/ cell |
| $G_o$ | 0 | | | 11 | 8.5 |
| | 100 | | | 17 | 5.6 |
| | 200 | | | 13 | 10.2 |
| $S_2$ | 0 | 23 | 6.9 | 11 | 8.6 |
| | 100 | 23 | 10.6 | 22 | 10.0 |
| | 200 | | | 8 | 9.6 |
| $G_2$ | 0 | 23 | 6.9 | 11 | 8.1 |
| | 100 | 10 | 9.2 | 3 | 6.5 |
| | 200 | | | 11 | 10.8 |

ii. 160 μM BrdU

| | | Exp 1 | | Exp 3 | |
|---|---|---|---|---|---|
| $G_o$ | 0 | 9 | 10.6 | | |
| | 100 | 23 | 11.6 | | |
| $S_2$ | 0 | | | 15 | 10.3 |
| | 100 | | | 15 | 17.3 |

in $G_o$ or $S_2$, with a convincing increase in only one case (Exp. 3; Table I, 5, 11ii). The possible slight increase also seen after $G_2$ X-rays could be due to the inclusion of some cells that were actually irradiated in late S phase, and have a very short $G_2$ period. After 400 r or 800 r during $G_o$, only three or four cells were scoreable but these had SCE frequencies that were no higher than those in cultures exposed to 200 r. This may be a selection effect for survival of cells with low SCE frequencies (although this is unlikely in view of the much higher levels of SCEs seen in chemical mutagen-treated cells; see Section F), or it could show that cells that survive have fewer aberrations and a related lower level of SCE. Once again, as we shall see later, this is unlikely as SCE and aberration levels are not closely correlated. A saturation effect at these low SCE levels also seems improbable in view of the results of Perry and Evans (1975) where Chinese hamster cells, exposed to 200 r in the S phase before observation, had almost double the non-irradiated SCE frequency and the numbers of SCEs continued to rise with dose up to about 35 SCEs per cell at 800 r, the highest dose tested.

b. <u>SCE in relation to chromatid aberrations</u>. In the present experiments, chromatid gaps and breaks were also scored in cells exposed to X-rays in $S_2$ and $G_2$, (Fig. I, 5, 5), and their occurrence in dark or pale-stained chromatids noted, along with the presence or absence of SCE at the break site. Seventy four such breaks were seen in dark chromatids and 57 in pale chromatids. The latter figure is probably an

Fig. I, 5, 5.

Human lymphocyte treated with X-rays during G2, showing chromatid aberrations (arrows). It is clear that chromatid breaks are not necessarily accompanied by SCE.

underestimate as gaps are not so obvious in the lightly stained material. This is consistent with the results of Dewey and Humphrey (1965) and of Wolff and Bodycote (1975) who found that doubly-substituted chromatids were no more radiosensitive than the chromatids unifilarly-substituted with BrdU.

There is evidence that a proportion of X-ray induced chromatid aberrations that appear as apparently simple chromatid breaks are a consequence of incomplete exchange (intrachange) events; this was the basis of the Revell hypothesis (Revell, 1959) - i.e. that all aberrations were the result of failed exchange events rather than of breakage and subsequent reunion. This can be tested by scoring SCE at the sites of chromatid aberrations; of the 130 such aberrations scored in the present study, only 17 (13%) were accompanied by SCE. This is well below the proportion of 40% expected if chromatid breaks are all the result of incomplete exchanges as proposed by Revell (1959; 1974) and is in accord with the results of Heddle and Bodycote (1970) who used ARG to demonstrate that 6% of the deletions induced by $G_2$ irradiation, and 24% of those following X-rays given in S phase, were associated with SCE. The FPG technique has also been applied to X-irradiated Chinese hamster cells where 8% of chromatid deletions were accompanied by SCE (Wolff and Bodycote, 1975).

iii. In Vitro: X-irradiation of Ataxia Telangiectasia Cells

Lymphocytes from AT patients have been reported to show a disproportionate increase in aberration frequency after exposure to X-rays in $G_2$ (Rary et al., 1974) or to gamma rays in $G_o$ (Higurashi and Coren, 1973); and fibroblast survival after gamma irradiation is also abnormally low (Taylor et al., 1975).

Lymphocytes from the four AT patients identified here were treated with X-rays (200 r) in $G_o$ (at the time of culture initiation) or in the second in vitro S phase ($S_2$) (about 12 hours before fixation). The cultures also contained 25 μM BrdU and were incubated for 72 hours to allow scoring of SCE and aberrations on the same slides. Aberrations were scored in M1 and in M2 cells, and analysis of these FPG stained cells was therefore less detailed than in G-banded preparations from the routine cultures used for background aberration assessment. All types of aberrations were noted: chromatid gaps, breaks and interchanges, both quadriradials and triradials, incomplete and complete, minutes, chromosome fragments, dicentrics, rings and abnormal chromosomes resulting from translocations. The background level of aberrations in non-irradiated cultures was obtained by analysing G-banded as well as orcein-stained preparations and some translocations would probably be missed when scoring FPG stained cells, so that the figures for irradiated cultures may be artificially low, but this should apply equally both to the control and to AT cells so should not affect the relative proportions of damage.

TABLE I 5 12.

X- irradiation of AT lymphocytes:- Chromosome and Chromatid aberrations.

| Treatment | Culture | AT Patients | | | | | normal individual |
|---|---|---|---|---|---|---|---|
| | | FS | KS | DB | SB | AT Total | |
| Control (No X-ray no BrdU) | Cells scored | 60 | 60 | 60 | 57 | 237 | 100 |
| | B+C cells as % Total | 18 | 16.7 | 30 | 51 | 28 | 1 |
| 200r $G_o$ (25μM BrdU) | M1 Cells scored | 10 | 20 | 39 | 19 | 86 | 12 |
| | B+C cells as % Total | 10 | 45 | 76 | 79 | 62 | 33 |
| | Increase over control level | -8 | 28.3 | 46 | 28 | 34 | 32 |
| | M2 Cells scored | 31 | 30 | 48 | 7 | 116 | 50 |
| | B+C cells as % Total | 19 | 47 | 50 | 43 | 41 | 28 |
| | Increase over control level | 1 | 30.3 | 20 | -8 | 13 | 27 |
| 200r $S_2$ (25μM BrdU) | M1 Cells scored | 24 | 17 | 23 | 10 | 74 | 31 |
| | B+C cells as % Total | 42 | 29 | 70 | 60 | 50 | 23 |
| | Increase over control level | 14 | 12.3 | 40 | 9 | 22 | 22 |
| | M2 Cells scored | 50 | 36 | 29 | 12 | 127 | 50 |
| | B+C cells as % Total | 60 | 19 | 79 | 58 | 53 | 52 |
| | Increase over control level | 32 | 2.3 | 49 | 7 | 25 | 51 |

* "B" cells are those containing chromatid-type aberrations;

"C" cells are those containing chromosome-type aberrations.

As expected, the proportion of chromatid damage was much greater after irradiation in $S_2$ than in $G_o$. The total numbers of aberrations are shown in Table I, 5, 12. After $S_2$ irradiation there is slightly more damage in M2 than in M1 cells, in controls and in the pooled AT results. Since BrdU incorporation increases radiosensitivity (Djordevek and Szybalski, 1960) this may reflect increased susceptibility of the more highly BrdU-substituted cells, irradiated during their second in vitro S phase and seen as M2 cells, compared with those that had undergone only one cell cycle in vitro prior to irradiation. After $G_o$ irradiation, M2 cells from DB and SB showed a greatly reduced amount of damage compared with M1 cells (Table I, 5, 12), probably due to loss of unstable aberrations at mitosis, but cells from FS and KS behaved differently in this respect. It is apparent from Table I, 5, 12 that cells from the four AT patients differ in their response to X-irradiation, and the lower background frequency of aberrations in the two sisters FS and KS, compared with the male patients DB and SB, is also indicative of variations among AT patients in the characteristic responses of their cells.

When spontaneous levels of aberrations are taken into account, the increase in total aberrations in irradiated AT lymphocytes is no greater than that seen in normal cells (Table I, 5, 12), so that no disproportionate response is apparent. However, a more detailed analysis of the results does reveal some important differences between AT and normal cells.

a. $G_o$ Irradiation

1. Chromatid damage. No chromatid damage was seen in normal cells irradiated in $G_o$ yet substantial increases in chromatid aberration frequencies were seen in similarly treated AT cells at M1 (Table I, 5, 13). This is in agreement with the observations of Taylor et al (1976), who noted a large proportion of chromatid damage in AT cells subjected to gamma rays in $G_o$; but these authors also found a high frequency of interchanges in such cells, while I found only one triradial in an M2 cell from an AT culture. Taylor et al (1976) suggested that irradiation induced single strand lesions in $G_o$ chromosomes and that these subsequently gave rise to chromatid aberrations at the ensuing metaphase. The higher frequency of such aberrations in AT cells was thought to reflect faulty repair of the single stranded lesions. It is also possible that the postulated reduction in efficient repair might be due to a direct effect of radiation on repair enzymes, but most information available about radiation effects on cellular proteins (reviewed by Oleinick and Rustad, 1976) involves exposure to very much higher doses than the 200 r used in the present work and in the experiments by Taylor et al (1976).

2. Chromosome damage: deletions. The numbers of fragments that were not associated with rings or dicentrics and therefore were thought to result from chromosome deletions, were scored in BrdU-treated M1 cells from the four AT patients and the results compared with observations on non-irradiated 48-hour cultures without BrdU, since these contain predominantly

TABLE I 5 13.

Analysis of chromosome and chromatid aberrations in AT cells after $G_0$ X-irradiation.

| | | No X-rays; no BrdU | | | | | | 200rad in $G_0$; 25uM BrdU | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 48h culture | | | 72h culture | | | M1 | | | M2 | | |
| | | No. | cells | No./cell | No. | cells | No./cell | No. | cells | No./cell | No. | cells | No./cell |
| Chromatid Aberations | FS | 2 | 30 | 0.07 | 1 | 30 | 0.03 | 1 | 10 | 0.1 | 4 | 31 | 0.13 |
| | KS | 1 | 30 | 0.03 | 4 | 30 | 0.13 | 1 | 20 | 0.05 | 3 | 30 | 0.1 |
| | DB | 5 | 30 | 0.17 | 1 | 30 | 0.03 | 20 | 37 | 0.54 | 4 | 48 | 0.08 |
| | SB | 0 | 17 | 0 | 4 | 40 | 0.1 | 6 | 19 | 0.32 | 0 | 7 | 0 |
| | AT Total | 8 | 107 | 0.08 | 10 | 130 | 0.08 | 28 | 86 | 0.33 | 11 | 116 | 0.1 |
| | Normal Individual | 0 | 30 | 0 | 1 | 100 | 0.01 | 0 | 16 | 0 | 4 | 50 | 0.08 |
| Chromosome Deletions | FS | 0 | 30 | 0 | | | | 0 | 10 | 0 | | | |
| | KS | 3 | 30 | 0.1 | | | | 7 | 20 | 0.35 | | | |
| | DB | 10 | 30 | 0.33 | | | | 11 | 37 | 0.3 | | | |
| | SB | 3 | 17 | 0.18 | | | | 12 | 19 | 0.63 | | | |
| | AT Total | 15 | 107 | 0.14 | | | | 40 | 86 | 0.47 | | | |
| | Normal Individual | 0 | 30 | 0 | | | | 2 | 16 | 0.13 | | | |
| Dicentrics | FS | 0 | 30 | 0 | 0 | 30 | 0 | 0 | 10 | 0 | 1 | 31 | 0.03 |
| | KS | 0 | 30 | 0 | 0 | 30 | 0 | 3 | 20 | 0.15 | 4 | 30 | 0.13 |
| | DB | 0 | 30 | 0 | 0 | 30 | 0 | 6 | 37 | 0.16 | 10 | 48 | 0.21 |
| | SB | 0 | 17 | 0 | 1 | 40 | 0.03 | 2 | 19 | 0.11 | 1 | 7 | 0.14 |
| | AT Total | 0 | 107 | 0 | 1 | 130 | 0.008 | 11 | 86 | 0.13 | 16 | 116 | 0.14 |
| | Normal Individual | 0 | 30 | 0 | 0 | 100 | 0 | 1 | 16 | 0.06 | 2 | 50 | 0.04 |

M1 cells also. Cells in their first metaphase after $G_o$ irradiation were examined to avoid the problems of loss of acentric fragments during cell division. The increase in fragments in the irradiated cultures was much greater in cells from two AT patients (KS and SB) than in cells from the other two patients or in normal cells.

3. Chromosome damage: dicentrics. The large number of dicentrics in irradiated cells from DB (six in 37 M1 cells and 10 in 48 M2 cells; Table I, 5, 13) is striking, and I found a total of six dicentrics in 68 M2 cells from the other three patients, compared with two dicentrics out of 50 normal cells. This augmented rate of dicentric formation in AT cells irradiated in $G_o$ is in direct contrast with the results of Taylor and his colleagues (Taylor et al., 1976) and is seen in three out of the four patients, so that it is not a chance result on one individual. The data in Table I, 5, 13 show that cells from one patient, FS, did not have an exaggerated frequency of aberrations after irradiation although her clinical symptoms and spontaneous aberration level (Table I, 5, 6) are typical of AT.

b. $S_2$ Irradiation. Damage in M2 cells after irradiation in the S phase immediately prior to observation was scored and the results are shown in Table I, 5, 14, along with the 'baseline' levels found in 72 hour cultures exposed to neither BrdU nor X-rays. In comparing the results, the greater accuracy of detection of chromosome aberrations in G-banded cells (untreated) compared with treated, harlequin-stained cells, must be borne in mind.

TABLE I 5 14.

Aberrations in M2 cells after S2 irradiation of AT and normal cells.

| | | 72 h, no X-ray, no BrdU | | | M2 cells, 200r in S2, 25μM BrdU | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cells scored | Aberr-ations | Aberrns per cell | Cells scored | Aberr-ations | Aberrns per cell | Inc. over background |
| Chromosome Aberrations. | FS | 30 | 8 | 0.27 | 50 | 18 | 0.36 | 0.09 |
| | KS | 30 | 2 | 0.07 | 36 | 3 | 0.08 | 0.01 |
| | DB | 30 | 2 | 0.07 | 29 | 12 | 0.41 | 0.34 |
| | SB | 40 | 28 | 0.70 | 12 | 4 | 0.33 | -0.37 |
| | AT Total | 130 | 40 | 0.31 | 127 | 37 | 0.29 | -0.2 |
| | (AT Total-KS) | 100 | 38 | 0.38 | 91 | 34 | 0.37 | -0.01 |
| | Normal Individual | 100 | 0 | 0.00 | 50 | 11 | 0.22 | 0.22 |
| Chromatid Aberrations. | FS | 30 | 1 | 0.03 | 50 | 24 | 0.48 | 0.45 |
| | KS | 30 | 4 | 0.13 | 36 | 5 | 0.14 | 0.01 |
| | DB | 30 | 1 | 0.03 | 29 | 20 | 0.69 | 0.66 |
| | SB | 40 | 4 | 0.1 | 12 | 9 | 0.75 | 0.65 |
| | AT Total | 130 | 10 | 0.08 | 127 | 58 | 0.46 | 0.38 |
| | (AT Total -KS) | 100 | 6 | 0.06 | 91 | 53 | 0.58 | 0.52 |
| | Normal Individual | 100 | 1 | 0.01 | 50 | 23 | 0.46 | 0.45 |

1. Chromatid Damage. The total numbers of chromatid aberrations are increased markedly by $S_2$ irradiation (Table I, 5, 14) in both normal and AT cells, and in cells from the brothers SB and DB this increase is greater than than seen in normal cells, while cells from the other pair of AT patients have normal (FS) or smaller than normal (KS) increases.

2. Chromosome Aberrations. Chromosome-type breakage should not be increased by S phase X-irradiation, and the results in Table I, 5, 14 show very little X-ray induced change when we consider the total result from all four AT patients; however, a fairly large increase in chromosome-type damage occurred in one patient, DB, and in the normal individual. This result may be largely due to problems of classification of 'small fragments'. When such a fragment lies close to a chromosome end, or is aligned with a chromosome, it may be confidently classed as an isochromatid aberration; but if the fragment has no apparent 'parent' chromosome, it is not possible to say categorically whether it is the result of a chromatid- or a chromosome-type break. Such 'divorced' fragments were scored as chromosome breaks in all the cells for the sake of consistency, and they account for nearly all the chromosome damage scored in the irradiated cells from the normal individual, so that the result in Table I, 5, 14 is probably spurious. This error should be relatively constant throughout the scoring, however, so that the comparative results for AT and normal cells are not invalidated. The important point in the information from $S_2$ irradiation is

that while two AT patients do have disproportionate increases in chromatid-type damage, the other two do not show the same trend.

c. SCE in X-irradiated Ataxia Telangiectasia Cells.

Despite the high proportion of chromatid damage in AT cells after $G_0$ or $S_2$ X-irradiation, there is no corresponding increase in SCE after $G_0$ X-rays and only a slight increase in SCE frequency after $S_2$ irradiation (Table I, 5, 15). If the result for the three AT patients FS, KS and DB are pooled, the increase from an unirradiated level of 7.9 per cell to a frequency of 11.1 per cell is slightly greater than the increase from 9.6 per cell to 10.9 per cell in normal cells. The small number of cells available for scoring in the case of SB may be responsible for the very much more marked increase here. The disproportionate rise in chromatid-type damage in irradiated AT cells from some patients (see above) does not therefore have an associated SCE increase.

TABLE I 5 15.

SCE in AT lymphocytes after X- irradiation in $G_o$ or in $S_2$.

| Treatment | | Control | AT Patients | | | |
|---|---|---|---|---|---|---|
| | | | FS | KS | DB | SB |
| 25μM BrdU | Cells scored | 25 | 21 | 20 | 20 | 4 |
| | Ave. SCE/cell | 9.6 | 8 | 7.9 | 7.6 | 5 |
| 200rad $G_o$ | Cells scored | 33 | 31 | 30 | 45 | 7 |
| | Ave. SCE/cell | 8.4 | 8.2 | 7.2 | 6.7 | 4.4 |
| 200rad $S_2$ | Cells scored | 50 | 50 | 36 | 29 | 9 |
| | Ave. SCE/cell | 10.9 | 11.6 | 10.9 | 10.4 | 13.4 |

## E. ULTRA-VIOLET-INDUCED DAMAGE

### i. Unscheduled DNA Synthesis: Xeroderma Pigmentosum

Cells from the photosensitive disease Xeroderma Pigmentosum (XP) show severely diminished excision repair of UV-damaged DNA (Cleaver, 1968) and an exaggerated number of chromosome aberrations in response to UV light (Parrington et al., 1971). A primary fibroblast line established in this laboratory from a patient with clinical symptoms of XP was investigated for its capacity to carry out 'unscheduled DNA repair synthesis' (UDS) (Rasmussen and Painter, 1964) after UV irradiation, and the SCE frequency determined for comparison with normal cells.

Cells were grown on glass microscope slides as described in Chapter Two, and after reaching confluency were treated for 24 hours with medium containing only 1% serum, to reduce the numbers of cells in S phase. After UV treatment the cells were replaced in medium containing a high specific activity of tritiated thymidine (10 μCi/ml). Autoradiographs were made and exposed for one week, before staining, and counting the grain distribution over the nuclei. A preliminary experiment was carried out in which the cells were exposed to the UV lamp for 25 seconds (minimum dose about 150 erg per $mm^2$) and the results compared with those from a primary culture from a patient with Klinefelter's syndrome, treated in the same way and at the same culture 'passage level'. After the one-week autoradiograph exposure period, there were three categories of grain distribution:

cells with all-over heavy label due to incorporation of tritium during normal S phase semiconservative DNA synthesis (0.5 to 5% of the cells); unlabelled nuclei (only one or two per 100 cells); and cells with intermediate label, with about 80-100 grains per nucleus, which are probably indicative of UDS. Twenty randomly selected cells of the latter type from the XXY culture had an average grain count of 82, while the count for the putative XP cells was 84 per cell. On this evidence the 'XP' cells had normal UDS, but a series of higher UV doses were tested in the same way, and scored 'blind' by counting grains on 20 cells per slide, on coded slides. The results (Table I, 5, 16) are consistent with a reduction in efficiency of UDS 'repair' synthesis at higher UV doses (500 seconds UV, or a minimum of 2500 ergs per $mm^2$) where the granular, clumped appearance of the chromatin makes it difficult to discern silver grains; but the impression is that the putative XP cells were so badly damaged by these UV doses that they did not incorporate any labelled thymidine.

ii. <u>SCE in Xeroderma Pigmentosum Fibroblasts</u>

The fibroblast cultures were at passage level 10-11 and chromosome preparations were poor with very few dividing cells. The BrdU also appeared to be toxic to fibroblasts at the fairly high level of 50 μM that was used because the medium (Ham's F10) contains a high concentration of thymidine. However, some satisfactory preparations of metaphase cells grown on slides and given long hypotonic treatments (see Chapter Two) were obtained, and I was able to establish that the XP and XXY cells have the same background level of SCEs

TABLE I 5 16.

Unscheduled DNA synthesis after UV-irradiation of fibroblasts from suspected Xeroderma Pigmentosum patient and an individual with Klinefelter's syndrome.

| | UV dose (sec.) | Average no. of grains per nucleus | |
|---|---|---|---|
| | | ?XP | XXY |
| I | 0 | 3-5 (background) | |
| | 25 | 84 | 82 |
| II | 50 | 45 | 80 |
| | 100 | 70 | 90 |
| | 500 | damaged cells | 80 |
| | 750 | with clumped chromatin | 60 |

(12 per cell for 10-12 cells). No results were obtained for SCE frequencies after exposure to UV or to ethylmethanesulphonate (EMS).

## F. CHEMICAL MUTAGEN EFFECTS IN VITRO

### i. Effects of Chemical Mutagens on Normal Cells

The frequency of SCE detected by autoradiography was known to be increased by exposure to chemicals such as 4-NQO (4-Nitroquinoline-1-oxide), MNNG (N-methyl-$N^1$-nitro-N-nitrosoguanidine) and MMC (Mitomycin C). Kato (1974a) and Latt (1974b) later used the H 33258-BrdU technique to demonstrate the sensitivity of SCE levels in human cells to low concentrations of MMC (Latt, 1974b).

Three mutagens were tested: Mitomycin C (MMC), a cross-linking and bifunctional alkylating agent; Ethyl-Methane Sulphonate (EMS), a monofunctional alkylating agent; and Adriamycin (AM), a cytotoxic drug used in cancer chemotherapy and thought to be an intercalating agent. Since the stability of these compounds is variable, and EMS in particular is known to have a short half-life, the chemicals were added to the blood cultures for the last 24-27 hours of incubation to allow an average of one cell cycle to take place in the presence of the mutagen, allowing for some chemically induced delay. Some tests were also done on the effect of adding the mutagens 24 hours after the initiation of culture, so that the chemical was present for up to 51 hours. BrdU was present at 25 μM throughout the culture period. Preliminary experiments were carried out a) to determine the concentrations of mutagens that should be used and b) to examine the effect of adding $10^{-4}$ M deoxycytidine (dCyd) (Latt *et al.*, 1975) to try to increase the numbers of M2 cells

Fig. I, 5, 6a.

"Harlequin" chromosomes, showing multiple chromatid interchanges induced by the anti-cancer drug Adriamycin. The drug ($10^{-7}$M) was added to the culture during the final 27 hours of incubation.

Fig. I, 5, 6b.

The quadriradial figure formed by a homologous chromatid interchange between the C-band areas of two chromosomes 9 (left) is almost certainly a true case of somatic recombination induced by Mitomycin C (MMC), as the clear identification of individual chromatids in "harlequin" stained preparations has allowed us to see that somatic crossing-over does indeed occur.

CHROMATID INTERCHANGES INDUCED BY MMC

9–9

by counteracting the inhibitory effect of BrdU on ribonuclease reductase (Meuth and Green, 1974).

a. Aberrations induced by Chemical Mutagens. MMC and EMS caused mainly chromatid-type aberrations, including frequent chromatid interchanges typically induced by MMC. AM caused every conceivable type of aberration, as previously reported by Vig (1971) and Newsome and Littlefield (1975); but the striking feature of AM-treated cells was the presence of multiple chromatid interchanges (Fig. I, 5, 6a). AM appeared to be the most lymphocytotoxic of the three drugs and is indeed an effective anti-leukaemic agent.

b. SCE induced by Chemical Mutagens.

1. SCE frequency. The results in Table I, 5, 17 show the effects of MMC, EMS and AM on SCE after 24 or 48 hours' exposure, and the decreased proportion of M3 cells illustrates the slowing of cell growth caused by these compounds. The frequencies of SCE are dramatically increased by all three mutagens (Fig. I, 5, 7) and the dose-response curve for MMC illustrates the very steep rise in SCE frequency with increasing concentration of MMC (Fig. I, 5, 8). Clearly the monofunctional alkylating agent is less effective at inducing SCE than are the very potent agents MMC and AM.

2. SCE in C-bands. Although the well known effect of MMC on secondary constriction areas (Nowell, 1964; Morad et al., 1973) was clearly seen, with attenuations of chromosome 1 and particularly 9, and interchanges between the

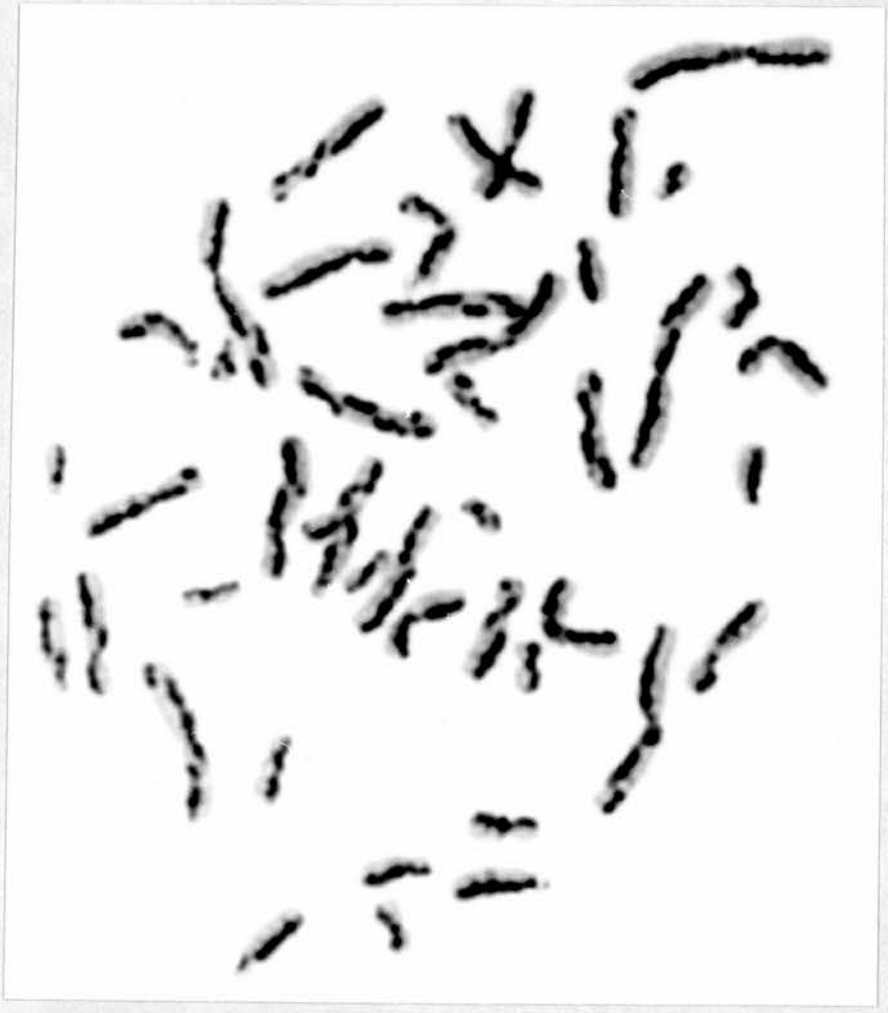


Fig. I, 5, 7*a*

"Harlequin" chromosomes, showing the dramatic increase in SCE frequency induced by Ethyl Methane Suphonate (EMS) in the presence of a very small amount of chromatid damage in the form of aberrations. The EMS ($10^{-3}$M) was present during the final 27 hours of incubation.

Fig. I, 5, 7b.

"Harlequin" chromosomes. showing a high frequency of SCE induced by Mitomycin C (MMC), and a low incidence of aberrations. The attenuated secondary constriction of chromosome 9 is a typical affect of MMC. The drug was added for the final 27 hours of incubation, at a final concentration of $3 \times 10^{-7}$M, and there are about 138 SCEs in this cell, compared with a background frequency of about 10 per cell.

Fig. I, 5, 8.

Induction of SCE by Mitomycin C.

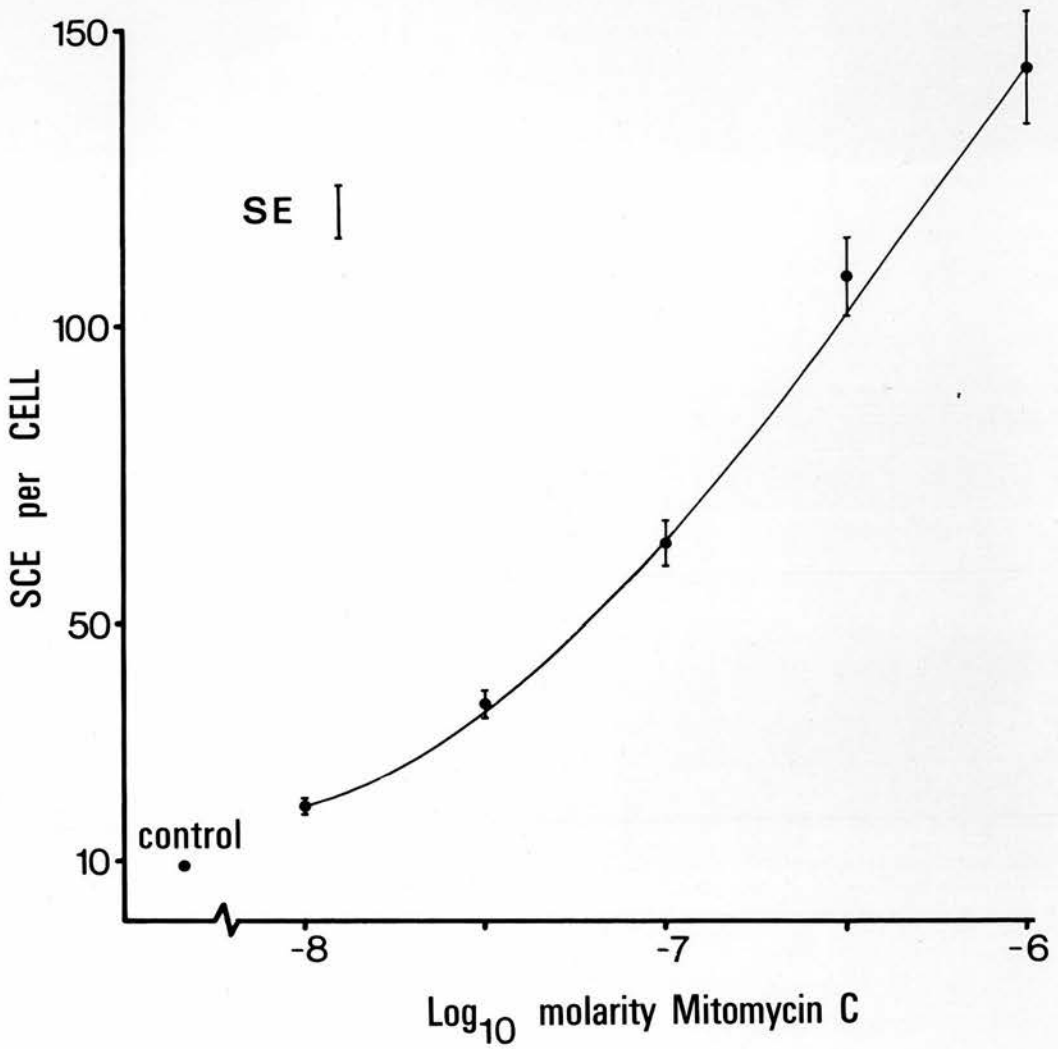


TABLE I 5 17.

Effect of three mutagens at two exposure lengths on SCE and proportions of M2 and M3 cells.

| | 25uM BrdU | MMC | | | EMS | | AM | |
|---|---|---|---|---|---|---|---|---|
| | | $10^{-8}$M | | $3x10^{-8}$M | $3x10^{-3}$M | | $10^{-7}$M | |
| | | 24h | 48h | 24h | 24h | 48h | 24h | 48h |
| SCE/Cell[a] | 10.9 | 17.5 | 24 | 28.5 | 51 | 73 | 18.6 | 26 |
| % M1 cells | 28 | 25 | 22 | 43 | 31 | 54 | 36 | 67 |
| % M2 cells[b] | 34 | 43 | 46 | 46 | 49 | 42 | 44 | 30 |
| % M3 cells[b] | 38 | 32 | 32 | 11 | 20 | 4 | 20 | 3 |

a. Average score for 30 cells

b. Proportions of a total of 100 to 350 mitotic cells per culture.

C-band areas of 1 and 9 (Fig. I, 5, 6b), the proportion of SCE in these areas was not increased except perhaps in 9 and the Y chromosome (see Chapter Three, Table I, 3, 6). The pattern of SCE induction in C-bands by EMS, AM and MMC is as follows:

EMS: Fewer SCE in the C-band of 1, but more in 9 and Y than controls (untreated cells).

AM and MMC: About the same proportion of SCE in 1, but more in 9, 16 and Y than controls.

3. Effect of deoxycytidine (dCyd). Deoxycytidine ($10^{-4}$ M) did not have a consistent effect on the proportion of M2 cells, but overall growth was slightly faster since the numbers of M1 cells were lower, and of M3 cells greater, than in cultures lacking dCyd. In contrast to the results of Latt et al (1975), who found that dCyd increased SCE levels, I found that the numbers of SCEs per cell in the presence and absence of dCyd were 10.6 and 11 respectively (average for 30 cells each). In cultures containing $10^{-8}$ M MMC, the SCE levels were 23.9 and 23.5 per cell with and without dCyd, respectively, so dCyd did not augment the SCE scores, and results from cultures with and without dCyd were pooled.

## ii. Effect of Chemical Mutagens on SCE Frequencies in Two Ataxia Telangiectasia Patients

Blood samples from the two brothers with AT (SB and DB) were cultured in the usual manner for 72 hours with 25 μM

TABLT I 5 18.

Effect of MMC, EMS and AM*1 on SCE frequencies in normal and Ataxia Telangiectasia cells.

(*1 MMC:Mitomycin C; EMS; Ethyl Methane Sulphonate; AM; Adriamycin )

| Treatment | | DB | SB | DB+SB | Control |
|---|---|---|---|---|---|
| $2.5\times10^{-5}$M BrdU | a | 6 (39) | 10 (43) | 8 (82) | 10 (102) |
| | b | | | 3-17 | 3-17 |
| MMC $10^{-7}$M | a | 58 ( 8) | ( 0) | 58 ( 8) | 74 ( 34) |
| | b | | | 16-73 | 30-120 |
| | c | x9.7 | | x9.7 | x7.4 |
| $3\times10^{-7}$M | a | 110 (53) | 107 (4) | 110 (57) | 92 (58) |
| | b | | | 60-155 | 37-140 |
| | c | x18.2 | x10.6 | x13.8 | x9.2 |
| EMS $3\times10^{-4}$M | a | 12 (26) | 15 (32) | 14 (58) | 18 (29) |
| | b | | | 5-35 | 12-24 |
| | c | x2 | x1.5 | x1.8 | x1.8 |
| | 51h*2 | 15 (56) | 17 (35) | 16 (91) | 15 (75) |
| $10^{-3}$M | a | 18 (34) | 25 (20) | 21 (54) | 27 (55) |
| | b | | | 9-54 | 17-38 |
| | c | x3 | x2.5 | x2.6 | x2.7 |
| AM $10^{-7}$M | a | 13 (25) | 23 (11) | 16.4 (36) | 24 (55) |
| | b | | | 9-29 | 8-44 |
| | c | x2.2 | x2.3 | x2.0 | x2.4 |

a Ave. SCE/cell (cells scored) b range of SCE scores per cell

c. factor by which SCE level is increased over baseline (25μM BrdU) level .

*2 Mutagens present for last 27h except in this one experiment, (51h).

BrdU, with chemical mutagens added for the final 27 or 51 hours of culture. Duplicate cultures with or without 100 μM ($10^{-4}$ M) dCyd were also set up, but because dCyd did not affect SCE frequencies (see above) the results were pooled.

a SCE frequency. Table I, 5, 18 shows the effect of MMC, EMS and AM on SCE frequency in cells from AT patients and a normal individual. Results on cells exposed for 51 hours were obtained only for EMS at $3 \times 10^{-4}$ M, and it is not known how long this unstable compound would remain active in culture. It is clear that in AT lymphocytes, the increase in SCE in response to these three different types of chemicals was just as great as that in normal cells. When treated with $3 \times 10^{-7}$ M MMC, AT cells seemed to show an even greater increase than normal cells, expecially in the case of cells from DB in which the baseline SCE frequency was low; but the level of 92 SCE per cell for the normal subject is lower here than in two other identical experiments, so this result may not be significant. The number of cells scored at $10^{-7}$ M MMC on this occasion was too low to refute or confirm this impression of an unusually high response in AT, but the results generally point to a normal response.

These observations are interesting in the light of results published recently by Latt et al (1975), where the SCE response of Fanconi's anaemia (FA) lympohcytes to MMC was markedly reduced compared with that of normal cells, and there was also a slightly lowered response to EMS. However, this inability to form a normal number of SCEs after

mutagen exposure was much less marked in FA fibroblasts, and it was suggested (Prof. S. Wolff, personal communication) that FUdR, present in Latt's lymphocyte cultures but not added to the fibroblast growth medium, might affect the SCE levels in lymphocytes.

b Effect of 5-Fluorodeoxyuridine on Mutagen-Induced SCE Levels. I was able to obtain further blood samples from DB and SB and also from the sisters KS and FS, to test the effect of 5-Fluorodeoxyuridine (FUdR) on their SCE response to MMC. The samples were cultured with 25 μM BrdU, 0.4 μM FUdR and 6 μM deoxyuridine (UdR). The results are shown in Table I, 5, 19, but since FUdR is rather toxic (Chapter Four, B, ii) no results were obtained for cells from DB and SB in cultures containing FUdR,and there were very few dividing cells even in control cultures on this occasion. The SCE data confirm that the response to MMC of AT cells from both these families is just as effective as that of normal cells, when the lower baseline frequencies of SCE in AT are taken into account. In cells from one patient, FS, FUdR did seem to supress the SCE response to MMC but the results on cells from her sister, KS, showed the reverse effect. These results do not necessarily invalidate the suggestion that FUdR may in some situations affect the SCE response to chemical mutagens, since AT patients even from the same families have been shown to differ in their characteristic sensitivities to certain chemical mutagens (Hoar and Sargent, 1976).

In normal cells, FUdR did not affect the SCE response to MMC, although there was a slight increase in SCE in control

TABLE I 5 19.

Effect of FUdR on SCE response of AT lymphocytes.

| Treatment | | Average SCE/Cell[a] | | | | |
|---|---|---|---|---|---|---|
| | | control | AT patients | | | |
| | | | FS | KS | DB | SB |
| 25μM BrdU | +FUdR | 11.8 | 8.9 | 8.3 | - | - |
| | -FUdR | 9.6 | 8.0 | 7.9 | 7.6 | (4)[b] |
| $3x10^{-8}$M MMC | +FUdR | 31 | 22 | 29 | - | - |
| | -FUdR | 32.2 | 30.6 | 25 | 26.3 | 25.8 |

a. Results averaged from 25-40 cells

b. Results averaged from only 3.5 cells.

cultures containing BrdU, FUdR and UdR compared with those containing BrdU only. This had been observed previously (Chapter Four, B, ii) and is probably due to more efficient incorporation of BrdU into the DNA of cells treated with FUdR to inhibit thymidine metabolism.

iii. Effect of Caffeine on SCE Frequency in Human Lymphocytes.

Since it seemed that SCE might be in some way associated with post-replication repair (PRR), and caffeine was thought to affect this type of repair process in mammalian cells (see Introduction to this Chapter), it was an interesting proposition to test the effect of caffeine on SCE frequency in human cells, and on the response to chemical mutagens.

a. Caffeine post-treatment. The results of two separate experiments are shown in Table I, 5, 20. Mitomycin C (MMC) was added 24 hours before fixation, and cultures washed with phosphate-buffered saline before addition of normal or caffeine-containing medium for the final seven or 12 hours of incubation. Table I, 5, 20i shows that there is a slight tendency towards an increase in SCE frequency caused by caffeine alone, and the effect of MMC is potentiated. In experiment II, where SCE from cycles One and Two, and from cycle Three, were scored separately in M3 cells, third cycle SCE were increased to a greater extent by the longer caffeine treatment of 12 hours compared with a seven-hour exposure. (Since SCEs in the third cycle occur in chromosomes that are either complete harlequins (visible SCEs) or completely pale, we can score only a proportion of the SCEs, and assume that

TABLE I 5 20

Effect of Caffiene post-treatment
on frequency of SCE and chromatid breakage

i.

| | | Average SCE/Cell[a] | | | |
|---|---|---|---|---|---|
| | | 1mM Caffeine | | No Caffeine | |
| | | 12h | 7h | 12h | 7h |
| I $3 \times 10^{-8}$M MMC | M2 | 23 | | 19.5 | |
| Control | M2 | 12 | | 8.1 | |
| II $1.2 \times 10^{-8}$M MMC | M2 | 21 | 22 | | 16.9 |
| | M3* | 13 | 8.4 | | 8.5 |
| Control | M2 | 7.6 | 9.5 | 8.7 | |
| | M3* | 3.4 | 2.8 | 2.8 | |

ii.

| | Chromatid breaks/cell[b] | | |
|---|---|---|---|
| | Caffeine (12h) | | -Caffeine |
| | 1mM | 5mM | |
| $3 \times 10^{-8}$M MMC M1+M2+M3 | 0.133 | 0.87 | 0.033 |
| Control M1+M2+M3 | 0.097 | | 0 |

a. Results from 18-28 cells

b. Results on 30-31 cells

* Visible third-cycle SCE only (i.e. approximately half actual total since about half the third-cycle SCEs occur between two pale-staining chromatids and cannot be detected.)

on average, half the third cycle SCEs are being scored).

These are very preliminary results and the timing of the treatment, and interactions between caffeine and different types of mutagens need thorough investigation. The effect of caffeine on SCE frequency in cells given MMC was confirmed in experiment I by a duplicate culture given a 5 mM caffeine treatment, where the SCE frequency was 27 SCE per cell with 11 visible third cycle SCEs per cell, compared with 23 per cell at 1 mM caffeine and 8.8 visible third cycle SCEs per cell. However, in BrdU control cultures (without MMC), the 5 mM caffeine did not give a further increase in SCE compared with 1 mM caffeine.

Chromatid aberrations (mainly gaps with some breaks and one interchange) were also scored, and Table I, 5, 20ii shows that 1 mM caffeine alone caused some aberrations, and that caffeine has a marked effect on the frequency of MMC-induced aberrations, especially at 5 mM caffeine, where the frequency of breaks is increased 26-fold.

It is clear that in human lymphocytes, caffeine has a potentiating effect on induction of aberrations by MMC, just as, in D6 Chinese hamster cells, Kato has demonstrated synergism of caffeine with UV radiation (Kato, 1974a). However, the experiments described here show that caffeine augments not only aberration levels, but also the frequency of SCE induced by MMC, whereas in the D6 Chinese hamster cells caffeine caused a depression of MMC-induced SCE. This

illustrates the problems of extrapolation of results from one cell type to form any hypothesis about mammalian cells in general (see below) and suggests that <u>if</u> post-replication repair (PRR) is indeed caffeine-sensitive in human lymphocytes, then this type of repair is not directly involved in the production of SCE; but the results suggest that PRR may be a factor in prevention of chromatid-type damage since interference with the postulated PRR mechanisms leads to larger numbers of chromatid aberrations.

## G. DISCUSSION

The central problem being investigated by these experiments is essentially that of the relationships among the mechanisms of SCE and chromosome aberration production, and repair processes. The experiments described above show that sex, age and chromosome constitution do not affect the background SCE level in human lymphocytes, and that cells containing gross chromosomal aberrations do not necessarily have high numbers of SCEs. It also seems that SCE is not the direct result of any one known repair system.

### i. SCE and Chromosome Aberrations

SCE are all reciprocal and symmetrical and occur between homologous sites, unlike chromosome aberrations, which may be asymmetrical and do not usually occur at homologous sites, except possibly in some of the aberrations seen in Bloom's syndrome (BS) cells, and cells treated with Mitomycin C. The evidence for a lack of a simple correlation between SCE and chromosome aberrations comes from several sources. The simplest cases are the chromosome instability syndromes AT and FA, where high spontaneous levels of damage are not accompanied by raised SCE levels, yet in Bloom's syndrome there are drastically exaggerated numbers of SCEs under the same conditions (Chaganti *et al.*, 1974). SCE is not an integral step in aberration formation since exchanges appear at the sites of only a proportion of chromosome breaks and

rearrangements, and in BS cells, for example, the locations of interchanges and SCEs are not related (Schroeder, 1975). Other information on SCE and aberrations comes from experimentally induced chromosome damage. The data in the present work show that in normal cells exposed to X-ray doses large enough to produce aberrations in up to 30% of the cells, levels of BrdU-detected SCEs are not greatly increased, yet chemical mutagens, at concentrations that cause only mild amounts of chromosome damage expressed as aberrations, can multiply SCE levels several-fold. Further indications that SCE and chromosome aberrations have different origins came from recent experiments on cells from Xeroderma Pigmentosum (XP) patients; although the alkylating agents EMS and MMS induce similar numbers of aberrations in XP and in normal cells, the XP cells develop exaggerated numbers of SCE in response to these chemicals (S. Wolff, personal communication).

In attempting to explain the differences in responses to different mutagens, we should consider the nature of the damage and of the repair induced. The early studies by Sax (1940) on chromosome breakage by ionising radiation suggested that broken chromosome ends could undergo restitution, or rejoining to give aberrations, and led to the theory, developed from the results of fractionated-dose experiments, that aberrations occur as a result of repair or mis-repair of broken chromosomes (Evans, 1962; Elkind and Sutton, 1959). A variety of repair mechanisms is recruited to deal with the individual types of damage

produced by ionising radiations, UV, and chemicals such as alkylating agents (reviewed by Regan and Setlow, 1973), and it seemed possible that SCE might be more closely connected with a type of repair involved in correcting chemical damage, rather than X-ray induced lesions, since this would account for the different magnitudes of SCE response to these different mutagens.

ii. Nature of the Lesions, and the Possible Relationships of Repair Systems to the Production of SCEs

The DNA lesions induced by mutagens are of many types. Ionising radiations produce large numbers of both single- (SS) and double- (DS) stranded breaks in the DNA, and some as yet poorly defined base damage. In the context of the type of repair mechanisms possibly involved in the generation of SCE, the alterations in DNA that are more important are lesions that alter the shape of the molecule, causing distortion and 'kinks' of the DNA backbone by inter- and intra-strand crosslinks, dimers, and base deletions or duplications in one strand. The repair mechanisms elicited to correct such damage in bacteria include excision repair (Fig. I,5,1) and post-replication or recombinational repair (Fig. I,5,2), which have been described in detail in the introduction to this chapter.

a. Results obtained with ultra-violet and ionising radiations. In cells exposed to UV (Griggs and Bender, 1973; Ikushima and Wolff, 1974) or to certain chemicals (Evans and Scott, 1969), aberrations are seen at the ensuing metaphase only if a period of DNA synthesis between mutagen-

treatment and observation has occurred, so that exposure during G2 does not result in aberrations immediately, but 'breaks' are seen after a further cell cycle. The lesions caused by ionising radiations, however, induce aberrations more directly, so that G2 irradiation does produce chromatid aberrations that are visible as soon as the cell reaches metaphase. SCEs, however, are not subject to the same rules, as G2 X-irradiation did not increase SCE at the subsequent mitosis (Perry and Evans, 1975), while X-irradiation in S and G1 caused a moderate rise in SCE frequency, illustrating the necessity for DNA synthesis before SCEs are visualised. All this suggests that the lesions giving rise to SCE are of a longer-lived type than the lesions predominantly involved in X-ray aberration production, and are less efficiently produced by X-rays than by chemical mutagens.

The major products of ionising radiation are single- (SS) and double- (DS) stranded breaks, while UV radiation induces pyrimidine dimers, especially thymine dimers, which are thought to be the main lesions leading to chromatid aberrations, since light exposure after UV treatment of toad cells abolished the normal UV-induced chromatid aberrations by photoreactivating the dimers (Griggs and Bender, 1973).

b. Results obtained with chemical mutagens. The damage produced by different classes of chemicals may take several forms. Experiments with the mono-functional alkylating agents MMS and MNNG showed that excision defective XP cells were capable of repairing this type of damage just as efficiently

as normal cells (Cleaver, 1971) and the results suggested that the agents produce single strand breaks directly by chemical depurination and hydrolysis. Although XP cells are defective in repairing UV damage, they can repair X-ray damage since ionising radiation produces single-stranded breaks directly and the initial incision step of excision repair is bypassed (Cleaver, 1968; Kleijer et al., 1970). The results obtained with MMS and MNNG are therefore similar to those from XP cells exposed to ionising radiation. In contrast, bi-functional alkylating agents and cross-linking agents cause intra-strand links and damage that must be removed enzymatically, so that the repair synthesis necessary to deal with products of these mutagens has kinetics similar to those in UV-irradiated cells.

Regan and Setlow (1973; 1974b) attempted to classify mutagens into two main groups according to the characteristics of the repair they induced: ionising-radiation-type damage, with rapid rejoining of SS breaks, and 'small patch' repair (MMS and EMS were assigned to this group); and UV-type damage, leading to slower 'large patch' repair following extensive excision and replacement, and induced by agents such as nitrogen mustard. There are complications in this system of classification as illustrated by some chemicals that induce both types of damage, such as 4-NQO, and the newer information on the effects of mutagens on cells from the chromosome instability syndromes shows that this classification of damage according to repair-type is an over-simplification. For example, AT cells, although

they are very sensitive to ionising radiation, are not hypersensitive to EMS, and FA cells, although very susceptible to MMC, are defective in excision of UV damage only after very high doses of UV (see Table I, 5, 21). Additional complications include the problem of metabolism of a chemical into different end-products, as in the case of MNNG. Although this chemical can alkylate DNA, the extent of this alkylation is not well correlated with its mutagenicity, so that the actual mutagen is thought to be a secondary product of MNNG in the cells (see review by Drake, 1969). There may well be overlap between repair mechanisms, following different types of damage, since some lesions are the common end-product of more than one type of mutagen (for example, dihydrothymine is a minor product of both UV and gamma irradiation [Remsenn and Cerutti, 1976]). It is not known how the cells deal with this damage. Although many interactions of, for example, alkylating agents with DNA are recognised, the particular reactions that are significant in generating transitions, transversions or frameshift mutations are not always known (see Drake, 1969), and the mutation may well be the result of faulty or 'error-prone' repair.

c. Nature of repair mechanisms. It has been suggested that SCEs reflect excision repair (Fig. I, 5, 1) (Kato, 1974b) or post-replication repair (PRR) (Fig. I, 5, 2) (Kato, 1973; Wolff et al., 1974; Bender et al., 1974). The hypothetical model was that SCE resulted from recombination between single poly-nucleotide strands (Gatti and Olivieri, 1973; Bender et al., 1974) (Fig. I, 4, 5) followed by a process of

strand migration (Sobel, 1972; Higgins et al., 1976) (Fig. I, 5, 9). The correlation with PRR was suggested by the depression in UV-induced SCE levels brought about by caffeine post-treatment (Kato, 1973) in Chinese hamster cells where PRR is caffeine sensitive; and later by the reduced SCE response to UV in XP variant cells where PRR is defective. The exaggerated numbers of UV-induced SCEs in XP also showed an interesting parallel to the situation in excision-defective E. Coli, where UV irradiation induced a higher frequency of recombination than in normal bacteria (Howard-Flanders and Boyce, 1966).

Although the precise significance of SCE in terms of its biological role is not clear, it is fairly certain that the events involved in generating these exchanges will involve the possibility of error and therefore of mutation and the fact that SCE can occur at the sites of gross aberrations, in some cases incomplete interchanges and chromatid breaks, shows that SCE can be incomplete or abnormal and therefore definitely associated with mutation. In bacteria (Witkin, 1969b) and meiotic yeast cells (Magni, 1963) mutation can arise from errors in recombination processes, so that if recombination is involved in SCE, it is possible that mutations may be associated with these exchanges.

Recently, several interesting papers have highlighted the difficulties involved in understanding repair mechanisms in eukaryotic cells. During replication of damaged DNA, gaps are left in DNA, presumably opposite lesions such as

dimers (Howard-Flanders et al., 1968), and in 1972 Lehmann showed that the gaps were filled in by a process that involved de novo DNA synthesis and not by insertion of a pre-synthesised strand as predicted by the prokaryotic model of recombination repair. Lehmann (1972) could find no evidence for recombination between DNA strands. It was later suggested that this 'replication repair' seemed to involve a process of branch migration and strand displacement, where a newly synthesised strand was used as an alternative template to damaged DNA, thus providing a detour around the lesion (Higgins, Kato and Strauss, 1976) (Fig., I, 5, 9).

However, other authors using similar density labelling techniques to those employed by Higgins et al (1976) found evidence for the appearance of hybrid DNA molecules in Chinese hamster cells (Fig. I, 5, 10), and for increased amounts of this DNA in cells treated with UV (Rommelaere and Miller-Faurès, 1976) or with Mitomycin C (Moore and Holliday, 1976). This type of molecule is a necessary step in models of recombination (Holliday, 1964) (Fig. I, 5, 10), and the amounts of the 'intermediate' hybrid DNA correlated well with the frequencies of SCEs induced by various doses of MMC (Moore and Holliday, 1976). Further evidence for recombination was obtained by experiments with an extremely sensitive technique, using human cells pulse labelled with tritium after UV irradiation, where the presence of pyrimidine dimers in daughter strands synthesised after irradiation was inferred from the existence of sites

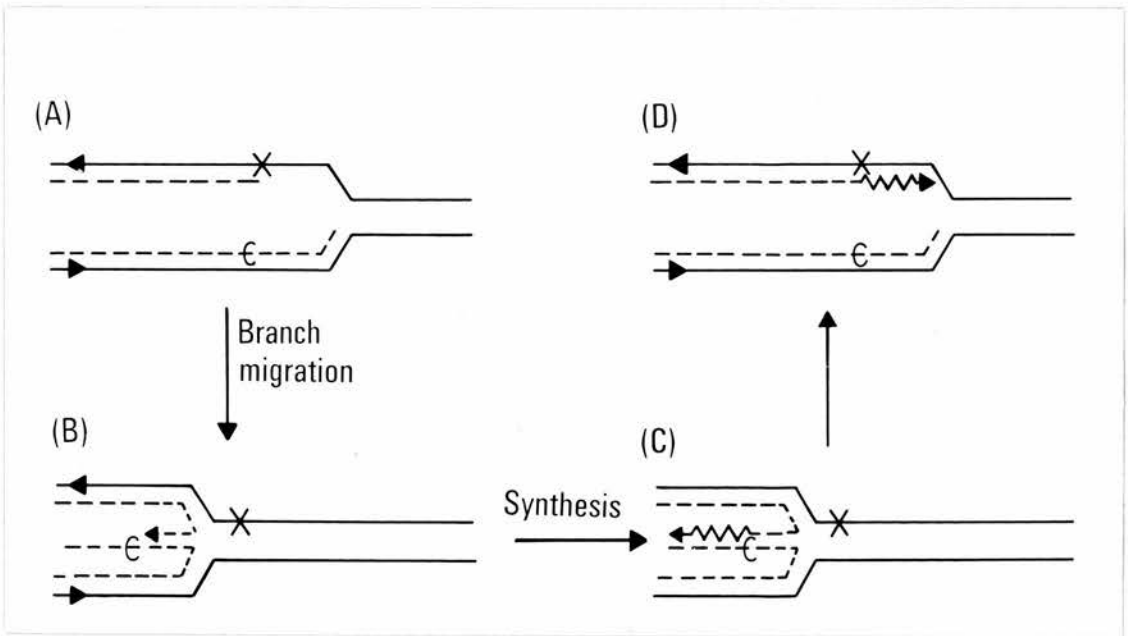



Fig. I, 5, 9.

Model of Replication Repair.

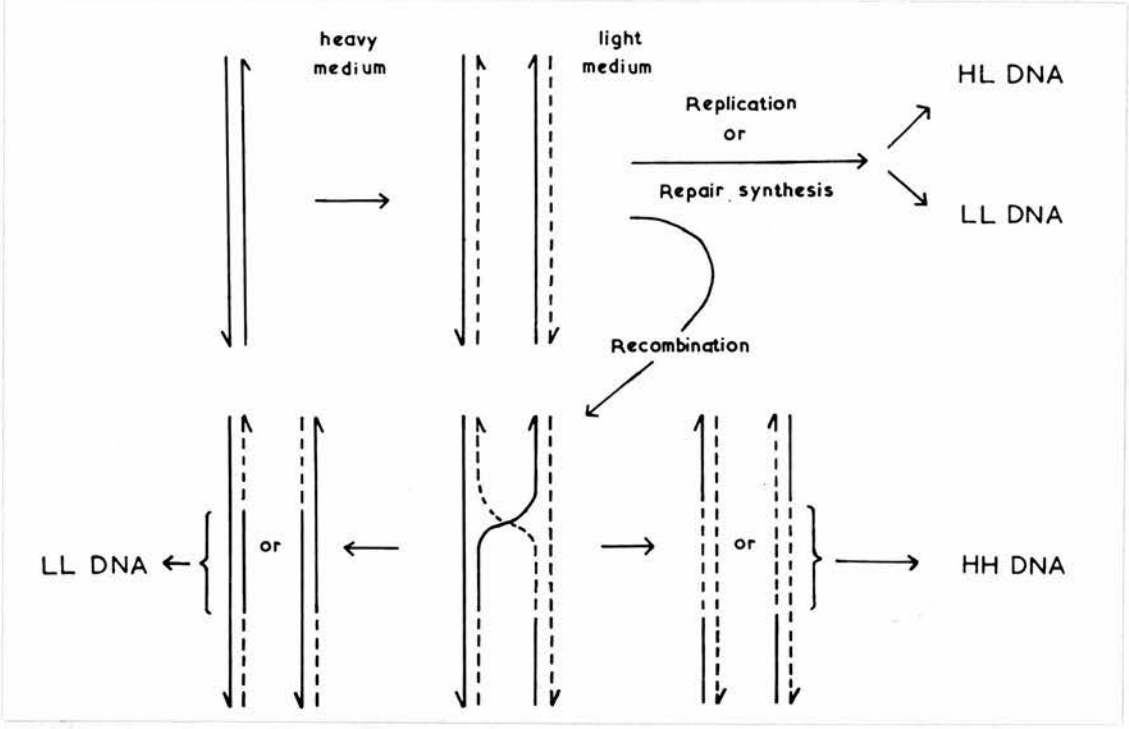
(after Higgins et al,1976).

Strand displacement and branch migration create an alternate template allowing replication to bypass a lesion (X).
This is an alternative model to the hypothesis that replication leaves gaps opposite lesions, which are later filled in by an unknown mechanism.

Fig. I, 5, 10.

Evidence for formation of hybrid DNA during mitotic recombination in Chinese hamster cells.

(from Moore and Holliday,1976).




Recombination is illustrated using the model proposed by Holliday,(1964), for meiotic recombination, but the recombinational repair model of Cole (1973; Fig. I, 5, 2 ) would give essentially the same result. Cells are labelled for less than one complete generation time with BrdU and tritium, so that one DNA strand is labelled or "heavy (H; dashed lines ). Cells are now returned to non-labelling medium (light, L ) so that any new synthesis can only lead to DNA that is half heavy (HL) or fully light (LL), unless recombination occurrs between chromatids, resulting in HH or LL "hybrid " DNA. The various classes of DNA molecules can be detected by their buoyant densities in neutral Caesium Chloride equilibrium gradients.

susceptible to a phage endonuclease known to 'nick' DNA at sites adjacent to dimers (Meneghini and Hanawalt, 1976). Evidence for the same phenomenon in XP cells had previously been published by Buhl and Regan (1973).

Evidently we need more conclusive evidence and descriptions of the molecular events involved in repair and recombination before we can draw any firm conclusions about the basis of SCE, but it is interesting that while the spontaneous levels of SCE are not altered in several types of repair-deficient cells examined so far, the one system where replication is abnormal and somatic recombination is frequent, namely Bloom's syndrome, also shows very high numbers of SCEs. This leads to another approach to investigations of repair defects and SCE, through studies of chromosome instability syndromes and repair-defective cells.

d. Chromosome instability syndromes, SCE and DNA repair. In Table I, 5, 21, I have attempted to summarise all the published data on the human repair-defective and chromosomal-breakage syndromes, in terms of mutagen sensitivities, specific repair defects, and incidence of SCEs.

1. Progeria. For comparison, the premature ageing syndrome, progeria, is of interest. There is some evidence that this infantile condition of accelerated ageing is inherited as an autosomal recessive (DeBurk, 1972), as are the chromosome breakage syndromes; but in the absence of published evidence to the contrary, it would seem that progeroid cells do not show chromosome instability. However,

TABLE I 5 21.

CHROMOSOME INSTABILITY SYNDROMES, XERODERMA PIGMENTOSUM and DOWN'S SYNDROME: MUTAGEN SENSITIVITY, REPAIR DEFECTS and SCE FREQUENCIES.

| | AT | FA | BS | XP | | DS |
|---|---|---|---|---|---|---|
| | | | | A-E | Var. | |
| Spont. Aberrations | | | | | | |
| Chromosome | + [1] | + [2] | + [3] | - [4] | - | - |
| Chromatid | + | ++ | ++ | - | - | - |
| Homol. Interch. | - | - [6] | ++ [3 6] | - | - | - |
| Spont. SCE Inc. | - [7 9] | - [8 11] | + [9] | - [10] | - [10] | - [11] |
| Inc'd Sensit. to:- | | | | | | |
| X-rays | + [12] | - [36] | | - [14 17] | | + [15] |
| γ rays | + [13 18] | + [13 37] | + [13] | - [14] | | + [16] |
| Anoxic γ rays | + [28] | | | + [20] | | |
| UV | ± [21 28] | + [19 24] | + [33] +/- [21] | ++ [4 17] | - [31 32] | + [15] |
| Alkyl. agents:- (a) | | | | | | |
| Monofunct. | + [23] | - [24 29] | | - [5] | | |
| Poly/bifunct. | + [23] | + [24] | | | | |
| Other chems. | + [23] | ++ [24] | | + [25 26] | | |
| Repair Defect. | | | | | | |
| DS rejoining (b | - [18] | | | | | |
| SS " (c | - [18] | | | | - [32] | |
| X-link removal (d | | + [24] | | | | |
| Excision:- | | | | | | |
| UV | - [28] | + [19] | - [33] | + [17] | - [31 32] | + [15] |
| Anoxic γ | + [28] | | | + [20] | | |
| γ | | + [38] | | | | |
| Post-Replicn. | | | | - [29] | | |
| Photoreactivn. | | | | + [34] | + [35] | |
| Increased Susceptibility to Cancer. | + | + | + | + | + | + |

a Alkylating agents, mono-, poly, or bi-functional

b Double-stranded break rejoining

c Single-stranded break rejoining

d DNA cross-link removal

TABLE I 5 21 : Bibliography.


1. Hecht et al, 1966
2. Schroeder et al, 1964
3. German, 1964b
4. Parrington et al, 1971
5. Cleaver,1971
6. Schroeder and German, 1974
7. Galloway and Evans,1975
8. Sperling et al, 1975
9. Chaganti et al, 1974
10. Wolff et al,1975
11. my unpublished results
12. Rary et al, 1974
13. Higurashi and Conen,1973
14. Kleijer et al,1970
15. Lambert et al, 1976
16. Sasaki et al,1970
17. Cleaver ,1968
18. Taylor et al,1975
19. Poon et al,1974
20. Setlow et al,1976
21. Cleaver,1968
22. Epstein et al,1973
23. Hoar and Sargent,1976
24. Sasaki and Tonomura,1973
25. Stich and San,1971
26. Stich et al,1973
27. Regan and Setlow,1973
28. Paterson et al,1976
29. Buhl et al,1972
30. Lehmann et al,1975
31. Burk et al,1971
32. Kleijer et al,1973
33. Gianelli et al,1976
34. Sutherland et al,1975
35. Sutherland and Oliver,1975
36. K. Buckton- unpublished data
37. Higurashi and Conen,1971
38. Remsen and Cerutti,1976
39. Finkelberg et al,1974


AT:- Ataxia Telangiectasia

FA:- Fanconi's Anaemia

BS:- Bloom's Syndrome

XP:- Xeroderma Pigmentosum; Complementation groups A-E and Variant

DS:- Down's Syndrome

Epstein et al (1973) have reported that progeria cells are defective in rejoining SS breaks after gamma irradiation. Although this report has been questioned by Regan and Setlow (1974a), who thought the findings were technical artefacts, Epstein and his colleagues were able to extend and confirm their results and the evidence for a repair defect in progeria seems fairly sound (Epstein et al., 1974). Added confirmation comes from the experiments of Brown et al (1976) who showed that progeroid cells were stimulated to carry out normal repair by co-cultivation with normal cells. There is some evidence that repair efficiency is decreased during normal ageing in vivo and in vitro (Lambert and Ringborg, 1976; Mattern and Cerutti, 1975) and that aberration induction by chemical mutagens is also exaggerated in individuals over 60 years old (Bochkov and Kuleshov, 1971), while viral transformation is more efficient in cells taken from older donors (Jensen et al., 1963) or cells that have become senescent in vitro (Todaro and Aaronson, 1968). On the other hand, repair efficiency of senescent fibroblasts is reduced only in the very last stages of culture degeneration (Goldstein, 1971; Painter et al., 1973) and the drop in repair replication does not appear until long after other senescent changes have taken place. Clearly UV-induced excision repair deficiency does not accompany in vitro ageing (Painter et al., 1973) but it is possible that other types of repair may be associated with senescence (Epstein et al., 1974; Mattern and Creutti, 1975). The present work has revealed no change in background SCE frequency with ageing, and it is interesting that in the adult premature ageing

condition known as Werner's syndrome, SCE levels are also normal (Bartram et al., 1976).

2. Less well-known chromosome instability and repair-defective syndromes. A rather less well-known example of chromosome instability than the syndromes AT, FA, and BS, is the disease Porokeratosis of Mibelli, where patients are also susceptible to malignant disease (Taylor et al., 1973). Cockayne syndrome may also prove to be informative, as it has some features of premature ageing along with sun-sensitivity, but chromosome stability appears to be normal, as with XP. However, the UV-sensitivity of these cells is increased, and unscheduled DNA synthesis decreased, although excision of pyrimidine dimers is normal (unpublished results of E. Chu and R. Schmickel, 1976).

3. Down's syndrome, AT, BS, FA and XP. A feature of chromosomally unstable cells which has not been included in the summary in Table I, 5, 21, is susceptibility to 'transformation' by viruses. Like senescent cells mentioned above (Jensen et al., 1963; Todaro and Aaronson, 1968), fibroblasts from Fanconi's Anaemia (Todaro et al., 1966) and Down's syndrome patients (Todaro and Martin, 1967) are more frequently transformed by SV40 virus than are normal cells and this could conceivably be related in some way to the increased incidence of malignancy in these individuals.

From the information presented in Table I, 5, 21, it may be seen that there are some discrepancies among results

of different studies of a given syndrome. For instance, the abnormal increase in aberrations after gamma irradiation of FA cells, reported by Higurashi and Conen (Table I, 5, 21; Ref. 37, 1971; 13, 1973) was not confirmed by Sasaki and Tonomura (Ref. 24, 1973) nor by the extensive study by Buckton et al (Ref. 36, unpublished data). Also, FA cells were not unusually sensitive to killing by gamma irradiation or by ethyl methane sulphonate (EMS; Ref. 39, 1974). This may point to genetic heterogeneity in FA, and indeed in 1976 Remsenn and Cerutti found that only two out of the four FA cases they examined had a reduced ability to excise gamma-ray-induced damage from exogenous DNA. There is, however, universal agreement on the extreme sensitivity of FA cells to MMC.

Some of the confusion in comparison of senstivities of particular cells to mutagens arises from the use of cell killing experiments in some laboratories and assessment of chromosome aberration levels in others, as these two factors may not of necessity be very closely correlated. This is further illustrated by the information that Bloom's syndrome cells, although they can repair UV damage (Cleaver, 1970) have decreased survival after UV irradiation (Gianelli et al., 1976).

From the Table (Table I, 5, 21) it is clear that background levels of BrdU-detected SCEs are not in themselves very revealing, but in response to mutagens they may be a rich source of information. In Micrococcus Radiodurans,

part of the increase in X-ray sensitivity following incorporation of BrdU is thought to result from inhibition of repair (probably excision repair) by the BrdU (Lett et al., 1970). If this occurred in mammalian cells, it might explain the lack of a dramatic effect of X-rays on SCE levels, assuming that SCEs do reflect some repair mechanism; but it is probable that the situation involved other factors, and a direct link between SCE and excision repair has not been demonstrated.

The spontaneous SCE level in both excision-defective and XP variant cells is normal (Wolff et al., 1975), yet an apparent parallel to the caffeine-induced depression of mutagen-induced SCE in Chinese hamster cells (Kato, 1974a) is found in the reduction in UV-induced SCE seen in cells of the XP variant, where PRR is known to be inefficient (de Weerd Kastelein, 1976). The defects in XP are not simply in PRR or in dimer excision, as reduced photoreactivation has recently been demonstrated (Sutherland and Oliver, 1975), and XP cells are also unable to excise a component of anoxic gamma ray damage (Setlow et al., 1976). It is also important to remember the complex background to repair, illustrated by the five complementation groups of XP that are all defective in the first, incision, step of excision repair. There is also evidence for genetic heterogeneity a) in FA, from the information discussed above, as well as from the variations in the age at onset of the disease and in severity of symptoms (Schroeder et al., 1976), and b) in AT, from variations in chemical mutagen sensitivities even between sibs, so that

the final levels of sensitivity seem to be a resultant of the AT genotype plus the parental sensitivity (Hoar and Sargent, 1976).

The conclusions that can be drawn using the information presented in Table I, 5, 21 can be summarised as follows:

i. Excision of UV damage is affected not only in XP, but also in FA and Down's syndrome cells;

ii. FA cells also show defects in excision of gamma-ray-induced damage and in removal of cross-links;

iii. Unlike XP cells, AT cells can excise UV damage effectively, yet both these syndromes involve an inability to remove some component of gamma-ray damage produced under anoxic conditions;

iv. Despite the sun-sensitivity shown by BS patients, BS cells can excise UV damage reasonably well.

Bloom's syndrome is still probably the most mysterious of these conditions, and the only available information is about not repair synthesis, but normal semi-conservative replication, and suggests that the depressed rate of replication (Hand and German, 1975) may be involved in the unusual features of BS cells (see Section iii, below).

e. Comparison of Responses of Different Cell Types. The ability of AT cells to develop SCEs in response to X-rays and three different classes of chemical mutagens seems unimpaired (this study), although in FA cells where excision of damage after high doses of UV, and repair of crosslinks induced by MMC are both reduced (Table I, 5, 21), there does seem to be a depression in the ability to produce SCEs in response to MMC, at least in FA lymphocytes (Latt et al., 1975). That FA fibroblasts, in contrast, had a nearly normal SCE response illustrates the care necessary in interpreting and comparing observations made on different cell types. The comparison between fibroblasts and lymphocytes in Latt's work (Latt et al., 1975) is complicated by the inclusion of FUdR and UdR in the growth medium used for lymphocytes but not for fibroblasts; but in the present study I found that FUdR did not have a consistent depressing effect on the SCE response of normal or AT lymphocytes to MMC. Following on the demonstration that human fibroblasts were more sensitive than lymphocytes to aberration induction by ionising radiation (Higurashi and Conen, 1971) or by the alkylating agent Trenimon (Arakaki and Schmid, 1971), it was shown that human fibroblasts also had higher levels of SCEs than lymphocytes after exposure to Trenimon, and that Chinese hamster fibroblasts were more sensitive still (Hayashi and Schmid, 1975b). In preparations made of cells from Chinese hamsters treated in vivo with butyl-nitrosourea, Engelhart (1976) has demonstrated the enhanced sensitivity of spermatogonia and marrow cells compared with lymphocytes.

In human lymphocytes the problem is further complicated by the existence of subpopulations of cells with differing radiosensitivities (Conard, 1969; Bender and Brewen, 1969; Steffen and Michalowski, 1973; Santos Mello et al., 1974; Janeway, 1975), and it is probable that such cells are also heterogeneous in their response to chemical mutagens (Abdou et al., 1973; Beek and Obe, 1974). There is some evidence that stimulation of lymphocytes by PHA renders them less sensitive to aberration induction by X-rays (Schreck, 1965; Conard, 1969) and MMS (Strauss and Coyle, 1968; cited by Spiegler and Norman, 1969), possibly as these more metabolically active cells have higher concentrations of repair enzymes; and indeed the uptake of tritium (used as an index of unscheduled DNA synthesis after UV irradiation) is increased by 50% in lymphocytes stimulated with PHA, compared with untreated lymphocytes (Darzynkiewicz, 1971). Lymphocytes stimulated physiologically have also been shown to have increased radioresistance, since T cells ('thymus-derived' cells) stimulated to blastogenesis in the graft-versus-host reaction are less sensitive to X-rays than unstimulated cells (Cauchi and Dawson, 1975). An important factor contributing to the heterogeneity of mutagen sensitivity may also be the variable growth rates of blood lymphocytes, illustrated by the proportions of M1, M2 and M3 cells seen in BrdU treated blood cultures, as cells may vary widely in their susceptibility throughout the cell cycle, and mutagens themselves perturb the cycle (Savage and Papworth, 1973).

In general, then, the cell type used for experiments

has a very important influence on the results, and this is further illustrated by the discrepancies between results of experiments with caffeine and alkylating agents carried out by Kihlman et al (1976) and by Kato (1974a). In Kato's studies on the D6 Chinese hamster cell line, the chemicals 4-NQO and MMC caused increases in aberrations and in SCE, but while the aberration numbers were exaggerated by caffeine post-treatment, SCE levels were reduced. This was taken as evidence that caffeine caused a reduction in PRR and a concomitant depression in SCE levels, and the situation in D6 cells seems to parallel that in human FA lymphocytes where the SCE response to MMC is low (Latt et al., 1975). However, Kihlman et al (1976) also used alkylating agents and caffeine to treat Chincese hamster or rat kangaroo cells, or root cells from the bean Vicia Faba, and found a caffeine-mediated potentiation of aberration induction, yet no effect on mutagen-induced SCE levels. It should be noted that the D6 Chinese hamster cell line tested by Kato is unusual in having a low background frequency of SCE, and was also reported to show no SCE response to MNNG (Kato, 1974b), although this mutagen is a potent inducer of SCE in Chinese hamster ovary (CHO) cells (Perry and Evans, 1975). It should perhaps be emphasised that in the experiments of Kato, the combination of the short half-life of MNNG and the use of autoradiography to detect SCEs, may make this result on D6 cells less convincing.

The brief experiments with caffeine carried out here on human lymphocytes do suggest that caffeine affects repair,

since aberrations and SCEs in normal or MMC-treated cells were increased by caffeine. Caffeine (1 mM) gave an additive effect with MMC, rather than showing the synergism reported by Brøgger (1974). The experiments were not directly comparable, however, since Brøgger added caffeine with the mutagens or immediately after irradiation, instead of removing the chemicals and giving the caffeine as a post-treatment for the final hours of incubation, as in the present work. The whole caffeine story is evidently very complex and there are as many different results and theories as experimentors, but more thorough investigations with well-characterised systems and fewer 'unknown quantities' should help to clarify our knowledge of the activity of this drug.

### iii. Aspects of Chromosone Structure and Behaviour: Possible Significance of the Observations on Cells from the Chromosome Instability Syndromes

The nature of the aberrations occurring spontaneously in cells from the chromosome instability syndromes, or induced by drugs such as MMC, has raised some interesting questions about chromosome structure and behaviour. The existence of many homologous interchanges in Bloom's syndrome (BS) cells has been used as evidence that there is somatic pairing of human chromosomes in interphase (Vogel and Schroeder, 1974). Although the random nature of chromosome involvement in aberrations induced by irradiation or occurring spontaneously in FA spoke against this theory, a specific timing effect was proposed to explain the discrepancies, postulating that chromosomes were paired during a crucial

period when the defect in BS or the activity of MMC, had their effects (Vogel and Schroeder, 1974). The aberrations induced by MMC have features in common with those seen in BS, although the MMC exchange sites involve preferentially the constitutive heterochromatin of chromosomes 1, 9 and 16 (Nowell, 1964; Morad et al., 1973), while BS aberrations involve many centric regions (German et al., 1974), and Comings (1975b) has suggested that the exchanges in both BS and MMC-treated cells are due to selection for exchange between regions of homologous DNA sequences, rather than to pairing of sister chromosomes. A defect in BS might therefore lead to longer exposure of 'open ends' or of single-stranded regions of the DNA, so that these were available for exchange; and indeed Hand and German (1975) have demonstrated a slower rate of DNA chain growth during normal semi-conservative DNA synthesis in Bloom's syndrome cells. The enzyme defect behind this is not clear and need not be a direct reduction in the amount or activity of DNA polymerase, as many other ineffective processes in metabolism could affect the rate of DNA synthesis; but a slower rate could conceivably lead to longer periods of DNA strand separation during replication, and thus increase the risk of exchange. So far, all the information from AT, FA and XP has been on repair synthesis, but these recent results show that normal replication is itself affected in BS and this may be important in the other peculiarity of BS, the spontaneous increase in SCE.

The use of the FPG technique leaves no doubt that the quadriradial configurations in BS do indicate somatic

recombination in these cells as German originally proposed (German, 1964b), since the positions of the chromatids are clearly seen. This was also demonstrated in the present work in FPG preparations of MMC- and AM-treated cells (Fig. I, 5, 6b). The biological significance of somatic crossing-over is hard to understand in mammals, and probably reveals abnormalities of DNA synthesis and repair, although this phenomenon occurs naturally in several systems and was first described in Drosophila 40 years ago (Stern, 1936). In fungi the generation of variability is a valuable product of somatic crossing over, but in most organisms no obvious selective advantage could result from this sort of rearrangement of the genetic material. A role in early embryonic development could be postulated, and certainly the association of BS with a high cancer risk could be linked with the production of abnormal cells by chromosome imbalance or mutation at exchange sites, or by the reassociation of deleterious genes. The latter process could lead to the generation of cells homozygous for recessive lethals and the resulting cell death may be involved in the retarded growth of BS individuals, both before and after birth. The study of such syndromes should continue to be a fruitful source of information and is important because of the hope it raises of finding out more about the fundamental enzyme processes associated with carcinogenesis. It is not valid to assume that all cancer is associated with a repair defect and Cleaver (1970) has quoted the example of the HeLa cell line, that was derived from a cervical carcinoma but has perfectly normal UV excision repair. It is nevertheless

possible that other types of so far unidentified repair defect may be present, leading to an increase in the somatic mutation rate, and possibly also involved in the increased susceptibility to virus transformation described above.

There are other genes, besides those for the chromosome instability syndromes and XP, that are associated with an increased cancer risk, and a genetic study of the relatives of FA patients (Swift, 1971; Hill, 1976) has shown that heterozygotes probably do have an increased tendency to develop malignancies, so that there may well be other genes that cause an increase in cancer risk in the heterozygote but are so far unrecognised because they are lethal in the homozygote.

### iv. Use of SCE as an Indicator of Exposure to Mutagens/Carcinogens

Clearly the question of the nature of SCEs is still unsolved and the molecular mechanisms behind these events require elucidation. Meantime, although SCEs are not sensitive indicators of X-ray damage, their induction by chemical mutagens *in vitro* may be used as a test system for mutagenicity of suspect compounds; and the sensitivity of SCE levels to chemical exposure *in vivo* may also be useful. Perry and Evans (1975) have used the FPG technique to detect increased SCE in lymphocytes from patients under treatment with cytotoxic drugs, and this system may become useful as part of a series of tests for monitoring exposure to harmful compounds in our environment, since SCEs are much more numerous

and easy to detect and score than are chromosome aberrations. They do, however, have the disadvantage that they can be visualised only in cells that have undergone two cell cycles in culture, and although there is evidence that some classes of lesions in DNA produced by a short 'pulse' of mutagen continue to induce SCEs during several cycles (Kato, 1974a), the increased SCE seen shortly after treatment in patients given cytotoxic drugs (Perry and Evans, 1975) falls fairly rapidly back to normal levels. This recovery effect was also obvious in the experiments on rabbit lymphocytes reported by Stetka and Wolff (1976; in press), and presumably reflects the rapid replacement of cells killed off by drugs, with descendants of stem cells in marrow and spleen which undergo several division cycles in a matter of perhaps 24 hours. After a further two cell cycles in vitro the SCE levels have returned to normal.

The uncertainty about the correlation of SCE events with mutation has been mentioned above (Chapter Five, Section ii, c); it is known that a proportion of SCEs can occur at the sites of gross chromosomal aberrations, and therefore SCE must be associated with mutation in these cases at least; and if recombinational processes are involved in generation of SCEs, then there is the possibility of errors such as the mutations detected in bacteria (Witkin, 1969b) and meiotic yeast cells (Magni, 1963). The results of this work are, however, consistent with the observation by Perry and Evans (1975) that "the ability of an alkylating agent to induce SCEs seems to bear no simple direct relationship to its

efficiency in inducing point mutations in bacteria, but is more closely related to its ability to induce chromosomal aberrations in eukaryotes." In other words, it is not valid to extrapolate conclusions from results of experiments with 'lower' cell types to apply to the complex systems in mammalian cells.

In conclusion, although the FPG technique has been used to clarify many issues, we are left with a variety of unanswered questions, both old and new, about SCE. We do not know for certain whether they always involve mutation, nor whether they are harmful to the cell, although SCEs seem to be relatively inocuous as cells can tolerate high numbers of them and continue to grow and divide. The use of SCE levels as a test system for mutagens/carcinogens will not be entirely justified until the molecular basis of the exchanges and their relationship to mutation are clarified, but studies of SCEs have already provided valuable information concerning aberrations and repair-deficiencies, and also about chromosome structure and behaviour.

# PART II

## INVESTIGATION OF AGE-RELATED CHANGES IN CHROMOSOMES OF BLOOD LYMPHOCYTES FROM A RANDOM SAMPLE OF THE POPULATION

# CHAPTER ONE

## INTRODUCTION

Ageing involves a very complex series of changes, and the reasons for the degeneration and loss of ability to renew tissues and cellular products have long been the subjects of much speculation. One serious problem is the question of whether ageing is seen truly at the level of the cell or of the organism, that is, whether cells deteriorate because they are old or because the organism is old (Maynard Smith, 1966). For example, do brain cells die because they undergo mutations and their organelles become damaged, or because of external factors such as changes in the vascular supply to the brain? The truth probably lies in an interaction between these two sorts of process.

### i. The Theory of Somatic Mutation

It is now clear that the incidence of many cancers increases with age (e.g. Doll, 1968). There have been many theories on the origin of cancer, including the implication of agents such as viruses or free radicals as carcinogens, but the most widely discussed concept is the theory of somatic mutation, proposed early this century by authors such as Boveri. The different theories are now seen to be perhaps complementary, as we now know that free radicals and viruses can interact at the genetic level. In 1951, Muller extended the theory of somatic mutation to the links between cancer and ageing, and suggested that an accumulation of somatic

mutations visible at the chromosomal level as aberrations was an important cause of ageing. This was further expanded in the 1950's (Failla, 1950; Curtis and Gebhard, 1958; Szilard, 1959) with the support of evidence such as the life-shortening effect of chronic irradiation in mice. However, there were inconsistencies, illustrated by the work of Curtis and Gebhard (1958), who showed that stresses other than radiation, such as chronic exposure to the toxic agents typhoid and tetanus vaccines, or the powerful mutagen Nitrogen Mustard, did not reduce the lifespan of the animals tested.

Since somatic mutation can be detected in chromosomes only by looking at dividing tissues, it is possible that the cells we examine are abnormal cells that are usually destroyed by selection, and that the situation in non-dividing tissues may be quite different. However, work on liver, usually in a non-dividing state but stimulated to regenerate by partial surgical excision or by treatment with carbon tetrachloride, has revealed the interesting fact that in mouse strains of characteristically shorter life spans, more chromosome aberrations appear than in similarly treated animals of longer-lived strains (Crowley and Curtis, 1963). This was confirmed in the guinea pig (Curtis and Miller, 1971) and is good evidence in favour of a link between the rate of somatic mutation and lifespan. Unfortunately, this rather artificial *in vivo* model is not very satisfactory for investigating normal ageing, and it seemed a simpler and better system was available when Hayflick and Moorhead (1961) described *in*

*vitro* senescence of cultured human fibroblasts. These cells were shown to have a limited survival time in culture, and chromosome aberrations accumulated as the cultures degenerated. It is still not clear if these aberrations are a cause or a result of the culture degeneration, and it is possible that the limited growth potential is simply due to our inability to provide the necessary conditions for longer term survival, since changes of treatment such as subculturing at altered ratios can vary the number of population doublings achieved (Hay, 1970). It is known that under one set of conditions the number of division cycles that fibroblasts may undergo *in vitro* is related to the age of the donor of the skin biopsy (Hayflick, 1965), so that embryonic cultures are capable of dividing a greater number of times than are adult fibroblasts. The survival time in culture also involves random events, however, since identically treated parallel cultures from one individual show wide variations in their *in vitro* survival times (Thompson and Holliday, 1973).

### ii. The Theory of Error Catastrophe

Related to the theory of somatic mutation is the idea that errors in various cellular processes lead to accumulating amounts of abnormal cell products and eventually result in cell death due to an 'error catastrophe' (Orgel, 1963). Substantial support for this hypothesis, essentially a theory of cytoplasmic ageing, comes from studies on alterations in proteins and nucleic acids of senescent tissue culture cells, both from normal individuals (e.g. Holliday and Tarrant, 1972; Bradley *et al.*, 1975), and from patients

with the form of premature ageing known as Werner's syndrome (Martin et al., 1965; Thompson and Holliday, 1973; Holliday et al., 1974; Goldstein and Moerman, 1975). However, there does not seem to be a general breakdown in accuracy of translation and protein synthesis since several viruses are known to be produced just as efficiently by degenerating cultured cells as by early passage-level cells, and there is no evidence for alterations in the viral structural proteins (Pitha et al., 1975; Holland et al., 1973).

If errors occurred in enzymes such as the polymerases involved in replication, DNA synthesis would be profoundly affected, and decreased rates of DNA synthesis in senescent cultured cells (Petes et al., 1974) and reduced fidelity of nucleotide incorporation by polymerase from liver of aged mice (Barton et al., 1974) suggest that this may well occur. Non-histone proteins (Maizel, 1975) and chromatin template activity (Ryan and Christofalo, 1975) are also altered in senescent cells, and growth is generally slower, but the importance of these events as causal changes in an ageing process is unclear.

iii Chromosome Changes during Ageing

a. Lymphocytes. The classical work on fibroblast survival in culture (Hayflick and Moorhead, 1961) was published at about the time that evidence was accumulating for an increase in chromosome aneuploidy in short term lymphocyte cultures from older individuals, first described by Jacobs and her colleagues in 1961. Later results revealed a sex difference

in the age effects on aneuploidy (Jacobs et al., 1963), showing that while the number of hypodiploid cells increased steadily in males the pattern in females fitted a cubic curve where there was a slow rise followed by a fast increase between the ages of 45 and 66 years, after which the proportion of hypodiploid cells rose more slowly again. It also appeared that there was a preferential loss or gain of a chromosome of the medium-sized or C-group in females, while a disproportionate number of male hypodiploid cells had lost a small acrocentric chromosome that could sometimes be identified as the Y, so that Jacobs and her colleagues suggested that the chromosome lost so commonly in female cells was possibly an X chromosome.

The reasons for these age effects, particularly obvious in females, are not known, and it is possible that chromosome loss may be random and the cells seen in culture have simply been selected by their ability to survive compared with cells lacking other chromosomes. Even if this were the case, some factor was causing cells from older individuals to lose chromosomes more frequently. It is interesting that in a study of 38 patients with cancers other than those affecting the reticulo-endothelial system, the age effect was found to be similar (Buckton et al., 1962) to that expected from the results of the 1961 study by Jacobs et al.

The first studies by the Edinburgh group (Jacobs et al., 1961; 1963) were carried out on a mixed group of individuals, including healthy volunteers, hospital patients and parents

of chromosomally abnormal children, but were later extended to a sample of the population selected randomly from the lists of general practitioners, in order to establish the normal frequencies of structural chromosome aberrations in the general population. The results published in 1964 (Jacobs et al., 1964) on 189 randomly selected subjects aged 65 years or more, bore out the conclusions of the earlier work and showed an increase in hypodiploid cells and a disproportionate loss of the putative sex chromosomes, although the increase in hyperdiploid cells this time was not significant. The study of 201 islanders of Tristan da Cunha (Hamerton et al., 1965) gave a similar picture, with an increase in aneuploidy, hypodiploid cells increasing at a rate that gave a fairly good fit to a cubic curve in females but was linear in males, and preferential loss or gain of C-group chromosomes in females. Another randomly selected population of 171 individuals of all ages in New York State was examined by Sandberg et al (1967) and a particularly marked level of hypodiploid cells in females over 60 years of age was found, but the increase in hypodiploid cells in males was not significant. Also, there was no steady increase in hypodiploid cells up to the age of 60 years so that Sandberg's data (1967) did not confirm the strong age-correlation reported by Jacobs et al (1964).

The nature of the increase in aneuploidy and the amount of the variation that is actually associated with age rather than other unknown factors remain in question, and although there is general agreement among reports that older individuals have higher proportions of hypodiploid cells (Goodman

et al., 1969; Jarvik and Kato, 1970; Jarvik et al., 1974; 1976; Mattevi and Salzano, 1975) there are some discrepancies in the details. For example, in the study by Jarvik and Kato (1970) the increase in female hypodiploid cells did not involve preferential loss of a C-group chromosome, while in males, although cells missing a small acrocentric chromosome did increase, there was no overall rise in hypodiploidy with age. The only exception among these studies is the report by Bloom et al (1967) where no age-related increase in aneuploidy was found in a population that was studied because of exposure to radiation after the explosion of the atomic bombs at Hiroshima and Nagasaki.

b. Other cell types.

All the above studies were carried out using peripheral blood lymphocytes, since it is not usually practical to examine other human tissues. However, after the report by O'Riordan et al in 1970 that the Y chromosome was sometimes missing in bone marrow preparations from three of the older subjects out of 32 males used as controls in a leukaemia study, a large study on marrow preparations was carried out by Pierre and Hoagland (1972). They found that an increasing number of males in older age groups had cells missing a Y chromosome and such cells were many times more frequent in marrow compared with blood, possibly since marrow cells divide so rapidly. An age-associated increase in aneuploidy of marrow of hamsters had also been reported by Hughes in 1968. On the other hand, Cadotte and Fraser did not confirm

the blood lymphocyte aneuploidy in their human marrow studies published in 1970; however, an examination of their results does show some increase in aneuploidy in marrow with age, which the authors considered insignificant.

A related phenomenon has been demonstrated in cultured human fibroblasts where cytogenetically marked clones were seen to develop in cultures of skin from adults and it seemed unlikely that these were simply a result of culture conditions since identically treated embryonic cultures did not develop clones of this sort (Harnden et al., 1976). The adult fibroblasts did not have a higher rate of other structural abnormalities, so that there did not seem to be a continuing instability of the chromosomes.

The information from fibroblast chromosome studies supports the theory of somatic mutation, although the significance of increased non-disjunction in lymphocytes and marrow is not clear, and increases in the numbers of cells with chromosome aberrations have not been established.

### iv. Aims of the Present Work - Part II

The purpose of the present study was to investigate age-associated changes in a random sample of the population, using G-banding for chromosome identification, since all previous reports had used conventional orcein staining and it was only surmise that the C-group chromosome apparently involved specifically in aneuploidy in females was indeed the X chromosome; and the role of the Y in aneuploidy in

males also needed elucidation. It was also of interest to identify the origin of the 'medium fragment',"an apparently acentric fragment the size of a C-group chromosome", which appeared with increasing frequency in the cells of older females, and occasionally in males. Moreover, if there was a disproportionate involvement in loss or gain of an X chromosome in female cells with increasing age, the new staining techniques to differentiate between the 'active' and 'inactive' X chromosomes could be utilised to find out whether the 'inactive' X was preferentially involved.

# CHAPTER TWO

## METHODS

### i. Subjects.

Chromosome studies were carried out on a random sample of 148 patients aged 20 years and over and identified by computer from the list of a general practitioner. Blood samples were obtained when the patient consulted his GP or attended a clinic at this hospital, or when a clinician from this Unit visited the subject at his or her place or work. Repeat samples were also obtained from some individuals, generally a year but in some cases 10 days to 18 months after the first sample. Additional samples were from groups of 14 year olds and people over 70 years old, also obtained through GPs. Usually the blood was taken from the elderly patients when a specimen was being sent for a haemoglobin estimation. Heelprick samples from newborn babies were obtained from the nursery at this hospital.

The random sample patients were also given a thorough medical examination and a battery of endocrinological and immunological investigations was carried out on the blood samples. Chromosome studies on these patients had previously been done as part of a survey of GP patients in this laboratory, but conventional orcein staining was used, so that this repeat study was carried out to investigate any chromosome changes more thoroughly by using G-banding.

ii. Culture Methods

Heparinised venous blood samples were cultured for two or three days using Ham's F10 medium with 10% bovine serum, PHA and antibiotics, and treated as described in Part I, Chapter Two.

Late Labelling

Methyl-$^{3}H$ thymidine at 0.2 to 0.5 μCi per ml, or BrdU at 25-650 μM was added for the last three to six hours of incubation. Autoradiographic and staining techniques have been described in Part I, Chapter Two. Autoradiographs were exposed for up to four months. G-banding was carried out using the ASG technique of Sumner et al (1971) and C-banding using the technique of Sumner (1972). Both these techniques are described in Part I, Chapter Two.

iii. Scoring and Cell Selection

An attempt was made to count 100 cells per person. Where possible 50 cells from two-day and 50 cells from three-day cultures were examined. Well-spread metaphase figures that looked regular in shape were selected using low power magnification, and before chromosomes were counted cells were examined at X 1000 under oil immersion to check that,

a) cells looked unbroken with no stray chromosomes in the same field, but with the chromosomes well enough separated for easy identification;

b) chromosomes had adequate G-banding (usually clearest in early metaphase cells where the chromosomes are fairly long and thin with sister chromatids lying parallel to each other);

c) centromeres were not splitting as a result of over-long exposure to Colcemide.

For each individual complete chromosome analysis was done on five cells, and on any cells with an aneuploid chromosome number or obvious abnormality. Remaining cells with a count of 46 chromosomes were simply recorded. The analysis of any abnormal cells was checked by a second observer in about half this work.

iv. Cell Classification

Cells were classified according to the system of Buckton (1962a) into types A, B and C.

A. Normal cells and cells with additional or missing chromosomes but no other abnormality detectable.

B. Cells containing chromatid aberrations such as chromatid gaps, breaks and interchanges, as well as isochromatid gaps. A chromatid aberration was classified as a break only if the distal segment was actually dislocated or lying on the wrong side of the chromosome.

C. These are cells with chromosome aberrations and are divided into:

Cs cells with potentially stable chromosome aberrations that survive mitosis, including rearrangements such as reciprocal translocations; and

Cu cells with 'unstable' aberrations such as rings, dicentrics or acentric fragments, which are less likely to be carried through successive cell divisions and may lead to breakdown of normal mitosis.

For purposes of analysing the data, cells were assigned to further categories as follows:

1. A and B cells: hypodiploid: 42-45 chromosomes
   hyperdiploid: 47 or 48 chromosomes

2. A + B + C cells: 'actual hypodiploid': a Cu cell of the type 45X + X fragment is not truly a hypodiploid cell as it apparently contains all the material for a diploid complement, so that cells of this sort are excluded from the total hypodiploid counts. Similarly the 'actual hyperdiploid' cells must include Cu cells of the type 46XX + X fragment.

3. The identity of any chromosomes lost or gained was noted, and cells were assigned to groups as 45-X;

45-(6-12); or 45-any other chromosome; also 45-21/22; and in males, 45-Y.

4. Cu cells were divided into those with and without 'medium fragments' and cells with these fragments were divided into those with replacement fragments, present instead of an X chromosome, or additional fragments, present besides a full complement of chromosomes.

v. Analysis of Data

For most purposes individuals of 20 and over were assigned to five-year age groups and newborn babies and 14-year olds were treated as separate groups.

Counts of abnormal cells were expressed as a percentage of the total number of cells counted for the individual, for each of 'early' (two-day) or 'late' (three-day) cultures.

a. Grouped data. For each category of cells, percentage values from the pooled data for each age group were plotted against mean age for the group. The numbers of cells scored were not the same for each age group so the data were analysed using a computer program that carried out weighted linear regression analyses, where the total numbers of cells scored for each group were used as the 'weights'. This method of analysis was chosen as the simplest way of testing for significant increases in aneuploidy with age, and although this gives no information on any change in the rate of increase, more complex forms of analysis are probably less valid since

the sample is so small with large individual variations. For the present linear regression analysis, where y = the percentage of abnormal cells and x = age, a better fit could be obtained by using the square root of y, and this is also necessary to avoid the problem of the increase in variance as the proportion of abnormal cells increases. A detailed explanation of this follows.

To estimate the proportion (P) of some population which has a particular characteristic, we can find the proportion (p) of the relevant characteristic in a random sample, of size N, from the population. From standard binomial theory:

$$\text{var } (p) \doteq \frac{P(1 - P)}{N}$$

If P is small then: $\text{var } (p) \doteq \frac{P}{N}$

In calculating the regression of p on some other variable for different populations, allowance must be made for the dependence of var (p) on P, since the standard least squares theory assumes that the variance is constant. The method is to use not p itself but a suitably chosen function of p. A standard result from statistical theory is:

$$\text{var } [f\ (p)] \doteq [f^1\ (p)]^2 \times \text{var } (p)$$

(see, for example, Rao [1952], Chapter Five)

Hence, if we use $f(p) = \sqrt{p}$, this becomes:

$$\text{Var}\left[\sqrt{p}\right] = \left[\tfrac{1}{2}p^{-\frac{1}{2}}\right]^2 \times \frac{p}{N} = \frac{1}{4N}$$

The use of a weighted regression analysis makes allowance for the size of N, so that we are left with the required condition of constant variance.

The significance of differences between regression coefficients for each set of data was tested as follows:

$$t = \frac{a_1 - a_2}{\sqrt{(SE_1^2 + SE_2^2)}}$$

where a = regression coefficient
SE = standard error

and the number of degrees of freedom is infinite.

b. Individual Data

To obtain a more detailed picture of the variations among individuals than is given by the use of the grouped data, the numbers of hypodiploid cells and of cells with unstable chromosome aberrations were also plotted for each individual against age, to give scatter diagrams.

# CHAPTER THREE

## RESULTS

### Sample Studied

Thirteen of the 148 blood cultures were not analysed, because of lack of metaphase figures or poor staining patterns, where G-banding would have been no more informative than orcein staining. One culture that failed to give a satisfactory result contained an extraordinarily high number of white blood cells. This was later found to be from a patient with Chronic Lymphatic Leukaemia (CLL) and a repeat sample was cultured using a very small amount of blood in each culture (0.1 ml) and dividing the final culture into four aliquots before hypotonic treatment to reduce the cell density; but despite these precautions only a very occasional metaphase cell was seen. This is in accord with the recent finding that CLL is associated with an abnormal proliferation of 'B lymphocytes' (or Bursa-derived cells), and it is thought that the cells that respond primarily to PHA, the mitogen used in our cultures, are 'T' or thymus processed cells (Wybran et al., 1973; Greaves et al., 1974).

Results were therefore obtained from 135 of the 148 random sample patients, 81 females and 54 males, and Tables II, 3, 1 and II, 3, 2 show the final composition of the analysed samples, along with the mean ages of each five-year age group and the total numbers of cells scored.

TABLE II 3 1.

Study Population;-Females.

| Age group | Subjects | Mean Age | Cells scored | | |
|---|---|---|---|---|---|
| | | | "Early" | "Late" | Total |
| Newborn | 8 | 0 | 300 | 175 | 475 |
| 14 | 2 | 14 | 100 | 88 | 188 |
| 20-24 | 3 | 22 | 150 | 155 | 305 |
| 25-29 | 2 | 28 | 100 | 100 | 200 |
| 30-34 | 9 | 31.8 | 350 | 450 | 800 |
| 35-39 | 6 | 36.8 | 250 | 350 | 600 |
| 40-44 | 6 | 41.8 | 150 | 450 | 600 |
| 45-49 | 7 | 46.9 | 200 | 450 | 650 |
| 50-54 | 10 | 52.2 | 550 | 451 | 1001 |
| 55-59 | 11 | 56.5 | 353 | 759 | 1112 |
| 60-64 | 11 | 62.3 | 50 | 970 | 1020 |
| 65-69 | 9 | 66.9 | 168 | 608 | 776 |
| 70-74 | 10 | 70.7 | 449 | 550 | 999 |
| 75-79 | 4 | 76.5 | 151 | 150 | 301 |
| 80-84 | 2 | 81 | 100 | 100 | 200 |
| 85-89 | 2 | 87 | 100 | 100 | 200 |
| Total | 102 | | 3521 | 5906 | 9427 |

"Early" ;- 2-day cultures

"Late" ;- 3-day cultures

TABLE II 3 2.

Study Population;- Males.

| Age group | Subjects | Mean Age | Cells scored | | |
|---|---|---|---|---|---|
| | | | "Early" | "Late" | Total |
| Newborn | 6 | 0 | 250 | 202 | 452 |
| 14 | 2 | 14 | 80 | 100 | 180 |
| 20-24 | 3 | 23 | 150 | 200 | 350 |
| 25-29 | 4 | 27.5 | 100 | 288 | 388 |
| 30-34 | 8 | 32.1 | 200 | 550 | 750 |
| 35-39 | 6 | 36 | 150 | 350 | 500 |
| 40-44 | 8 | 42.9 | 800 | 455 | 755 |
| 45-49 | 10 | 46.9 | 300 | 497 | 797 |
| 50-54 | 6 | 52.2 | 250 | 300 | 550 |
| 55-59 | 5 | 58 | 250 | 300 | 550 |
| 60-64 | 2 | 62 | 100 | 100 | 200 |
| 65-69 | 0 | - | - | - | - |
| 70-74 | 1 | 70 | 50 | 50 | 100 |
| 75-79 | 1 | 78 | 50 | 50 | 100 |
| 80-84 | 2 | 82 | 150 | 50 | 200 |
| 85-59 | 1 | 85 | 0 | 130 | 130 |
| Total | 65 | | 2380 | 3622 | 6002 |

"Early";- 2-day cultures

"Late" ;- 3-day cultures

## A. STRUCTURAL VARIATION

Variations in the size of satellites and their distance from the centromere were common, but were not classed as variants. C-banded preparations were examined in cases where the secondary constriction of chromosome 1, 9 or 16 appeared very long with G-banding, and it was noticeable that a very long pale region on a G-banded chromosome 9 did not necessarily correspond to a very large BaOH C-band. Of the 102 females, I found one individual with a large C-band on chromosome 1 (1 qh+), and one with 9 qh+, using the size criteria normally used in this laboratory; but C-banding was not carried out on cells from the whole sample. Of the 102 females, there were also three with a total inversion of the C-band of chromosome 9 (Fig. II, 3,*a*) and one with a partial inversion of this C-band. One previously identified case of a balanced translocation between chromosomes 11 and 17 was also included (MRC Registry number K203), and I found a Robertsonian translocation between chromosomes 13 and 14 in a woman who had not previously been karyotyped (K377). This translocation was also found in cells from the sister of the proband, but the phenotypically normal sons of both the translocation carriers were too young to karyotype.

In one individual, a 'fragile' chromosome 16 was noted. A high frequency of spontaneous breakage of specific chromosomes in cells from normal individuals has been reported by Gooch and Fischer (1969) and Giraud et al (1976), but the reason for the 'fragility' is not known. In the present

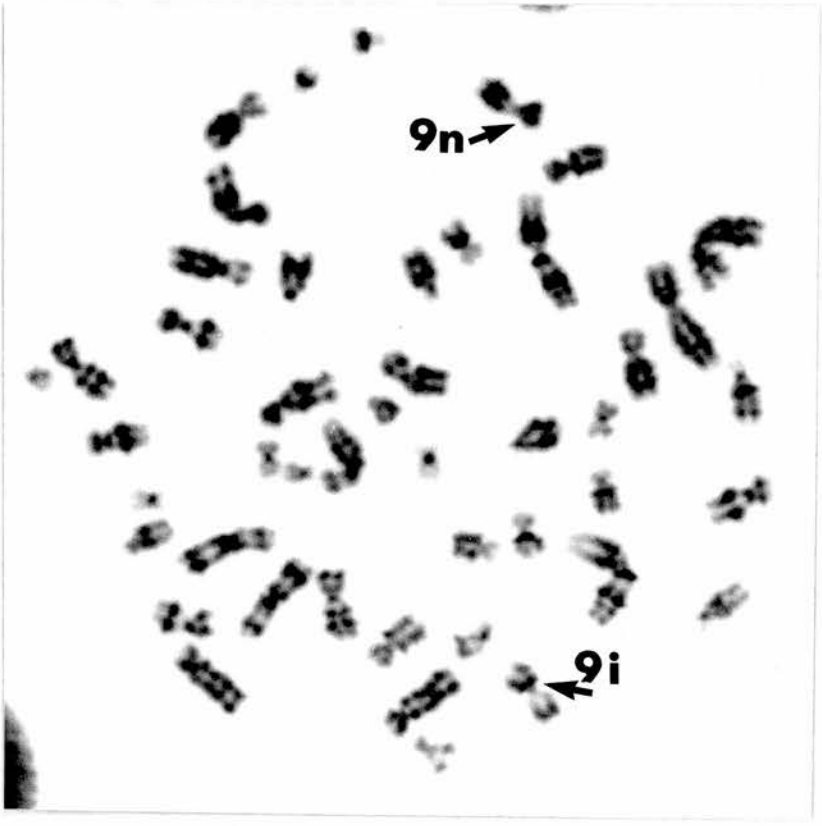



Fig. II, 3, a.

G- banded cell from a normal female, showing a total inversion of the C-band area of chromosome 9 (9i) , with the normal chromosome also indicated for comparison, (9n).

study, in 100 cells from a culture that had been given a late-labelling treatment with tritiated thymidine, I found three cells with a chromosome break, resulting in a deleted chromosome 16 and a fragment, and one cell with an isochromatid gap in chromosome 16 q13 or q21. However, a parallel culture without tritium also had three cells with chromatid breaks and three with chromosome breaks in chromosome 16, out of a total of 100 cells. This evidence that the chromosome was apparently spontaneously fragile was supported after the autoradiographs were exposed, and the cells containing the broken chromosome were found to be unlabelled, so that the breaks were not caused by the isotope. The break point in the chromosome did not always appear to be in exactly the same band but was always in 16 q11-3 or in 16 q21 - i.e. just below the C-band. Unfortunately all the cells scored were from preparations made from 72 hour 'late' cultures, so that the amount of breakage occurring after only one cell cycle in culture, and more likely to be a reflection of an *in vivo* situation, was not known. The significance of such specific chromosome fragility is not clear, but the report by Giraud *et al* (1976) states that 18 individuals with a 'fragile' chromosome (10, 12, 16 and 17) were found among the parents of chromosomally abnormal children, and in couples with histories of multiple spontaneous abortions. Phenotypic abnormalities were evident in the individuals concerned only when the chromosome involved was an X chromosome.

## B. ANEUPLOIDY: COMPARISON OF MALE AND FEMALE DATA

The complete data are shown in Appendix III. The results of the weighted linear regression analyses on all the data are summarised in Tables II, 3, 3a and II, 3, 3b, showing in which categories increases occurred that are significant by the criteria of this test. The full details of the regression analyses with the values of a and b for the equation

$$\sqrt{y} = a + bx$$

are given in appendices IV and V.

### i. Hypodiploid Cells

The information pooled from both two- (E) and three- (L) day cultures is shown in Fig. II, 3, 2, and the increase in hypodiploid cells with age is significant in both sexes (Table II, 3, 3), although the proportions are generally higher in females than in males ($t = 5.3$, $p < 0.001$). Between the ages of about 30 and 55 the amount of hypodiploidy remains at about 2-3% in males, and 3-5% in females; but the level rises steeply to about 8% hypodiploid cells at about 60 years in males and about 70 years in females. The apparent fall-off in hypodiploidy in very old individuals, and the drop from the newborn level to zero at 14 years, are probably largely due to low numbers of cells scored to obtain these results (Tables II, 3, 1 and 2).

### ii. Hyperdiploid Cells

Hyperdiploid cells show a significant increase in females

TABLE II 3 3.

Results of Weighted Linear Regression.

a. Females

| | hypo | A+B 45 | 45-X | 45-(6-12) | 45-other | hyper | Cu | Cu+X fr | other Cu | Cs | B+Cu+Cs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E | + | ++ | + | - | + | ++ | ++ | ++ | - | + | ++ |
| L | + | ++ | - | - | + | + | +++ | +++ | - | - | + |
| E+L | ++ | ++ | ++ | NT | NT | ++ | +++ | NT | - | + | NT |

b. Males

| | hypo | 45-Y | hyper | B+Cu+Cs |
|---|---|---|---|---|
| E | +/- | NT | -- | NT |
| L | +/- | NT | -- | NT |
| E+L | ++ | - | -- | + |

NT not tested

\- not significant

+++ highly significant

++ significant at 1% level

\+ significant at 5% level

+/- significant at 10% level

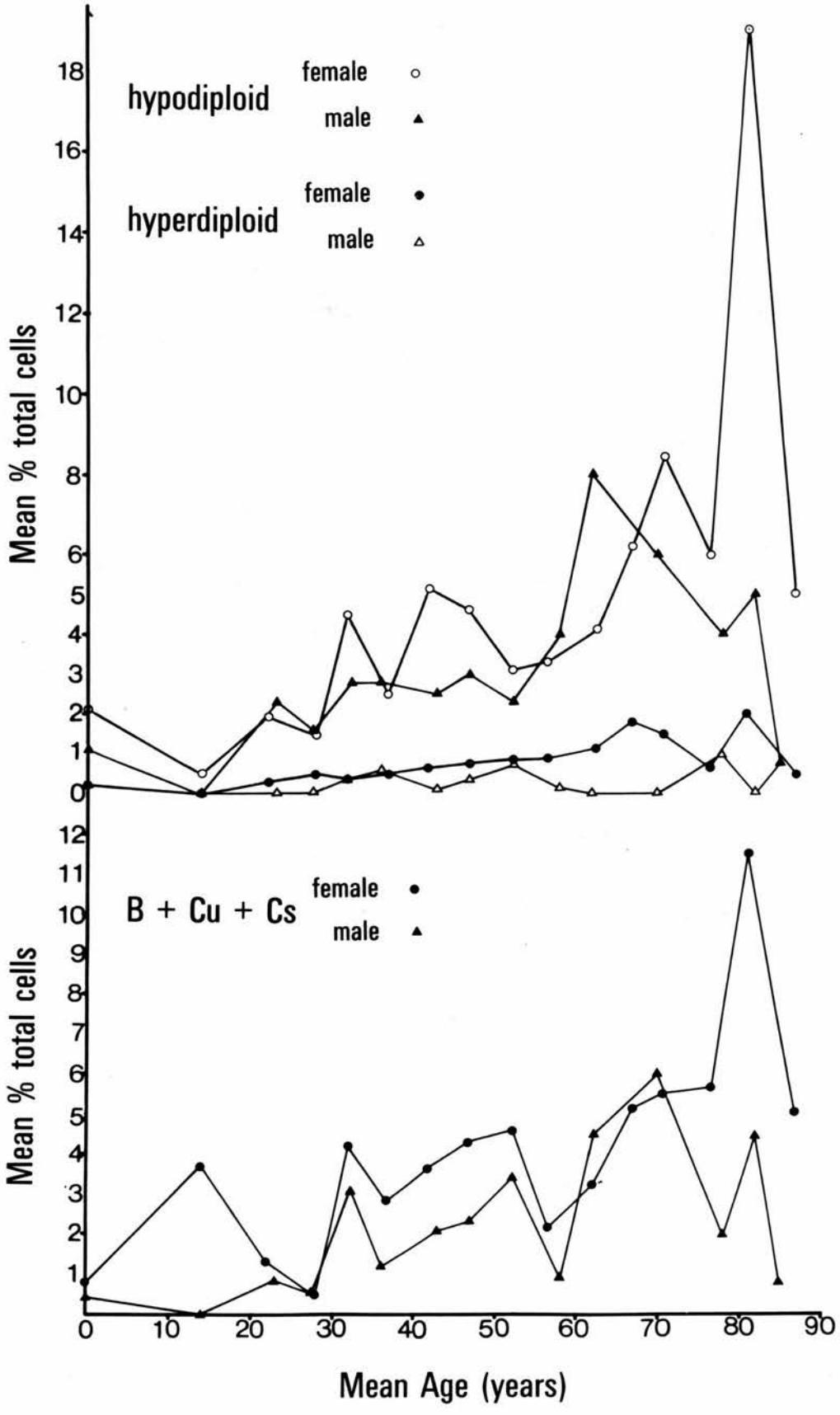
Aneuploidy; Total data for males and females.

Fig. II, 3, 2.

Proportions of hypodiploid and hyperdiploid cells for each age group, plotted against the mean age for each group.

Fig. II, 3, 3.

Proportions of cells with chromatid (B) or chromosome (unstable;-Cu, or stable, Cs) aberrations, plotted against the mean age per group.


hypodiploid
female
male
hyperdiploid
female
male
Mean % total cells
B + Cu + Cs
female
male
Mean % total cells
Mean Age (years)

but not in males, and these cells constitute only a small proportion of the total with a maximum of about 2% in females and less than 1% in males. The proportions of hyperdiploid cells are much lower than the levels of hypodiploid cells, both in males ($t = 5.3$, $p < 0.05$), and in females ($t = 1.89$, $p < 0.1$) and the overall frequency of hyperdiploid cells does not differ significantly between the sexes.

iii. Cells with Chromosome and Chromatid Aberrations

The proportions of B and C cells are presented in Fig. II, 3, 3 and these also show a significant increase in both sexes, with slightly higher proportions in females. Once again the rate of increase of aberrant cells is fairly slow until the upturn at about 60 years of age.

## C. ANALYSIS OF THE DATA FROM THE FEMALE SAMPLE

The complete data are shown in Appendices III, a, b and c.

### i. Hypodiploid Cells

Since the individual variations in proportions of abnormal cells are so great, and the graphs of pooled data show rather marked fluctuations, scatter diagrams of the data for each individual may give a more informative picture of any age effect in these results. Fig. II, 3, 4 is such a diagram, showing 'actual hypodiploid' cells (see Chapter Two, iv) from both 'early' and 'late' cultures for females. There is a general trend towards an increase in these cells with age, but the very wide range of values is clearly illustrated and a small number of only five individuals are contributing the highest value of over 12% hypodiploid cells at ages 67 and over. Below this scattergram the same information is plotted using the mean percentage of hypodiploid cells as before, and the mean age for each five year age group, but plotting the square root of y (where y = mean percentage of hypodiploid cells) to illustrate the fitted line calculated by the computer, using a weighted linear regression analysis (Fig. II, 3, 5). The numbers of cells scored to obtain each point (weights) are also shown to illustrate the relative importance of the fluctuations.

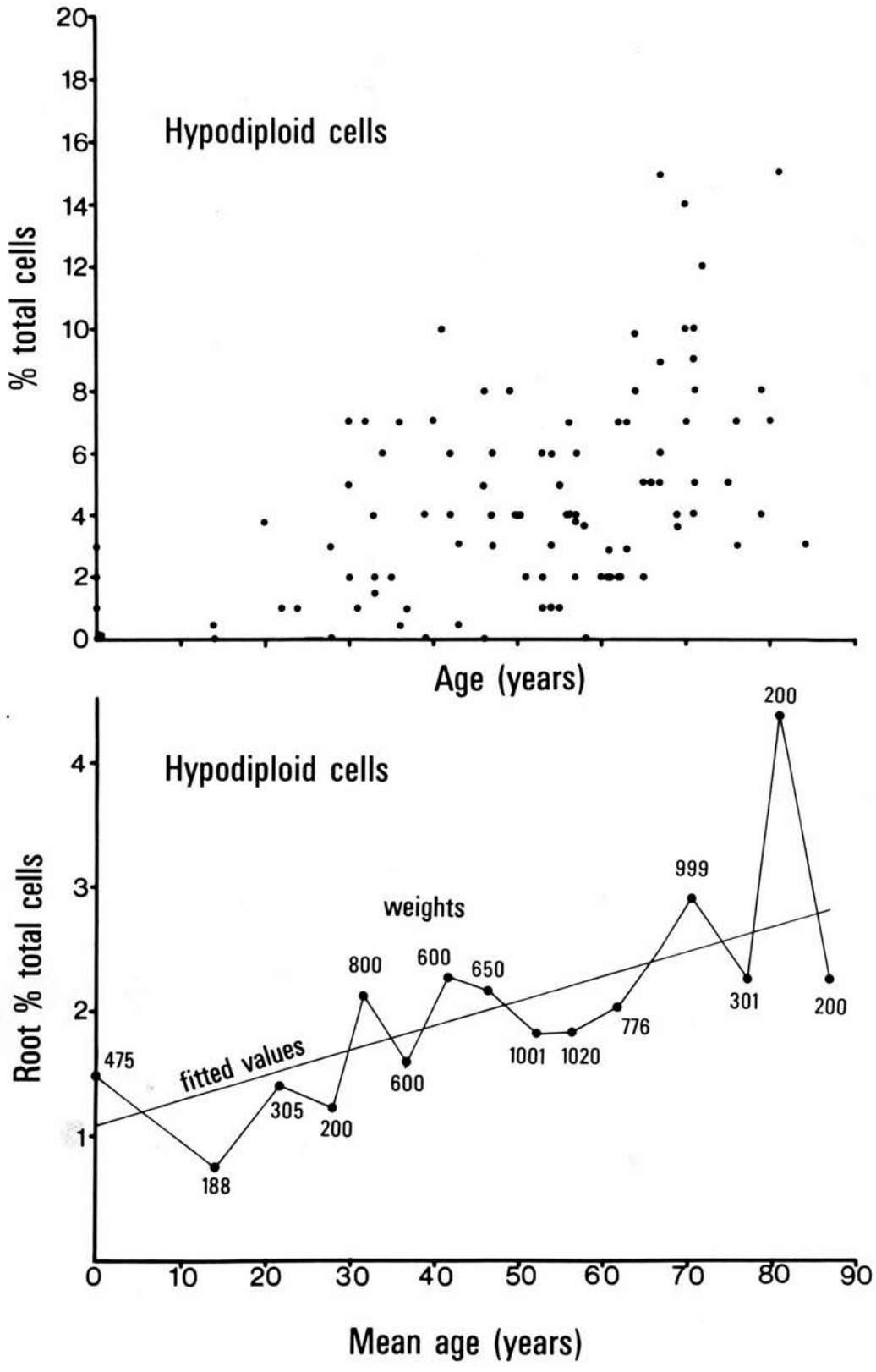
Hypodiploidy in Females.

Fig. II,3, 4.

Scattergram, showing the proportion of hypodiploid cells found in each female, plotted agaisnt the age of each individual.

Fig. II, 3, 5.

The square root of the mean percentage of hypodiploid cells is plotted against the mean age for each five-year age group, to illustrate the values used in the weighted linear regression analysis, and the fitted line derived from this analysis is also shown. The numbers of cells counted to obtain each point (weights) are also shown.


Hypodiploid cells
% total cells
Age (years)
Hypodiploid cells
Root % total cells
weights
fitted values
475
188
305
200
800
600
600
650
1001
1020
776
999
301
200
200
Mean age (years)

ii. Identification of Lost Chromosomes

The proportions of hypodiploid A and B cells - i.e. cells with no chromosome aberrations but with chromosomes missing - are plotted in Fig. II, 3, 6. Here it is clear that a large proportion of the cells with 45 chromosomes are missing an X chromosome, and that these make a greater contribution to the total than the sum of all the cells missing other medium-sized chromosomes (6-12) although the difference between these two regressions is not significant. Cells lacking chromosomes of the group 6-12 are less common than those missing any other chromosome ($t = 2.18$, $p<0.05$). The important role of (45-X) cells in the increase in hypodiploidy with age in females is convincingly demonstrated. The increases in (45-X) and (45-other chromosomes) cells are significant according to the regression analyses (Table II, 3, 3) but the numbers of cells of the [45-(6-12)] type are not significantly raised. The large peak of '45' cells seen at 81 years of age, and the sharp drop that follows at 87 years, are most probably due to the low numbers of cells (200 for each point) rather than to a real difference, and one of the two individuals scored for the last point on the graph had an unexpectedly low number of hypodiploid cells.

A detailed analysis of the chromosomes lost and gained in female cells is presented in Table II, 3, 4. The prominence of (45-X) and (47 XXX) cells (Fig. II, 3, b) in females is striking, and it is also interesting that chromosomes 8 and 9 are more frequently lost than others of this size group. Of the total of 259 cells with 45 chromosomes,

Fig. II, 3 b.

G- banded cell from a normal female aged 54 years. The cell contains 47 chromosomes, and the additional chromosome is an X.

## Female Hypodiploid Cells.

### Fig. II, 3, 6.

This graph shows the contribution to the total numbers of cells with 45 chromosomes, of cells lacking an X chromosome, any other "medium-sized" chromosome (6-12), or a chromosome of any other group.

### Fig. II, 3, 7.

The effect of time in culture on the final proportions of cells from females which have 45 chromosomes.

E; 2 day culture

L; 3 day culture.

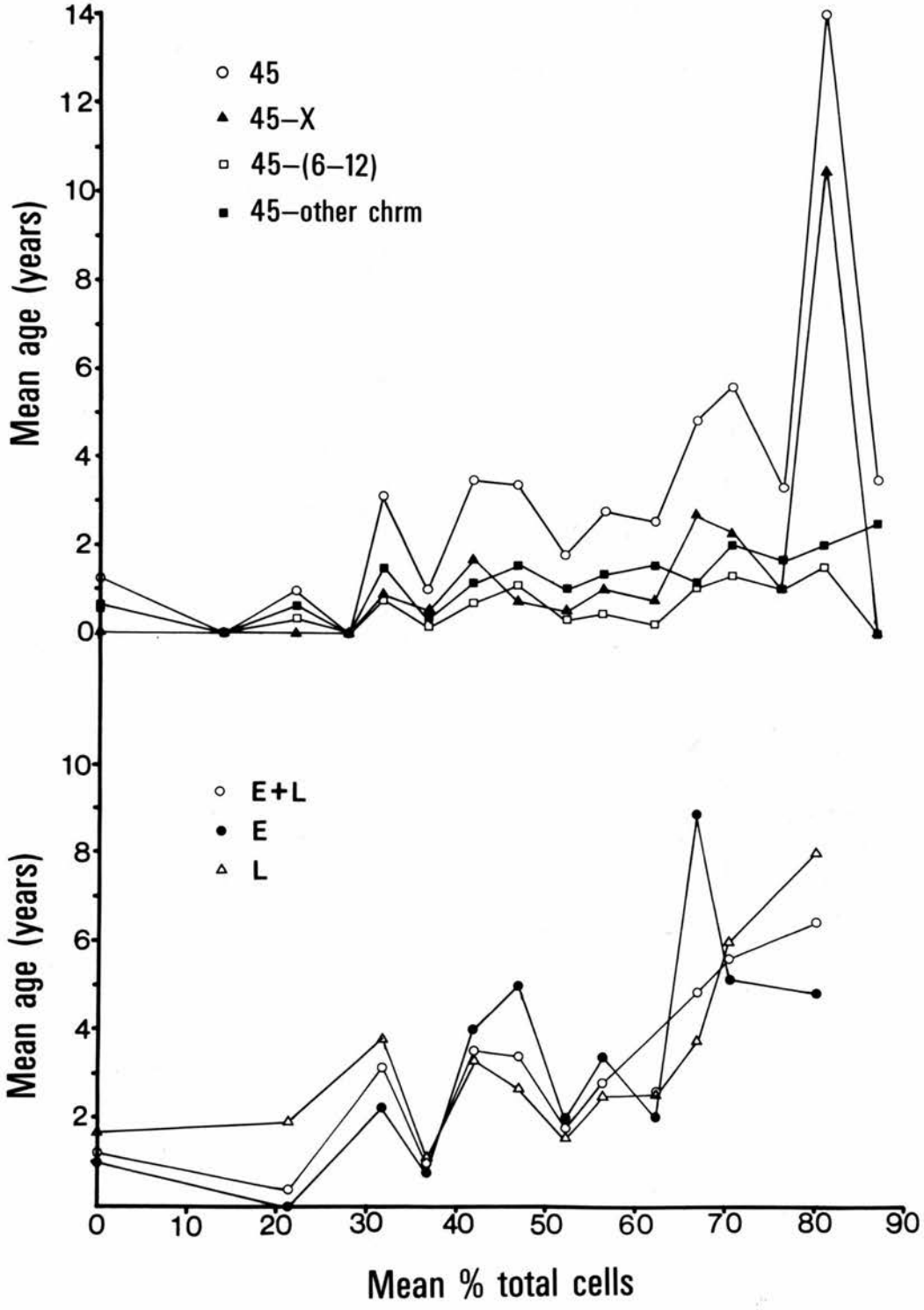

Mean age (years)
14
12
10
8
6
4
2
0
45
45–X
45–(6–12)
45–other chrm
Mean age (years)
10
8
6
4
2
E+L
E
L
0
10
20
30
40
50
60
70
80
90
Mean % total cells

TABLE II 3 4.

Chromosomes lost or gained in female or male cells with 45 or 47 chromosomes.

* E= expected value if loss random according to number of homologues

** chromosome groups where individual chromosomes were not identified

| Chromosome | Females | | Males | |
|---|---|---|---|---|
| | lost (E=10)* | gained (E=1)* | lost (E=3.9)* | gained |
| 1 | 4 | | 1 | |
| 2 | 1 | | 1 | |
| 3 | 7 | | 0 | |
| 4 | 4 | 1 | 2 | 1 |
| 5 | 1 | | 2 | |
| 6 | 3 | 1 | 1 | |
| 7 | 5 | 1 | 1 | 1 |
| 8 | 13 | 2 | 5 | |
| 9 | 9 | 2 | 2 | 1 |
| 10 | 3 | | 9 | 1 |
| 11 | 6 | | 3 | |
| 12 | 5 | | 2 | |
| X | 84 | 27 | 0(E=2)* | |
| 13 | 6 | | 3 | |
| 14 | 2 | 1 | 2 | 1 |
| 15 | 4 | | 3 | |
| 16 | 8 | 1 | 3 | 1 |
| 17 | 11 | 1 | 3 | |
| 18 | 6 | 1 | 7 | |
| 19 | 11 | 1 | 5 | |
| 20 | 7 | 2 | 6 | 1 |
| 21 | 19 | 3 | 11 | |
| 22 | 12 | 1 | 4 | |
| Y | | | 14 (E=2)* | |
| Total | 231 | 45 | 90 | 7 |
| ** B | 3 | | | |
| C | 18 | 6 | 2 | |
| D | 2 | 1 | 1 | |
| E | 1 | | | |
| F | 4 | | | |
| G | | 1 | | |

146, or 56%, have lost a medium-sized chromosome, when, if loss were random, the expected frequency (based on the numbers of these chromosomes without taking size into account) would be 16/46 - i.e. 35%. This is a significant difference by the chi-squared test (chi-squared = 4.8, p less than 0.05). In general, the smaller chromosomes also show a greater tendency to be lost or gained than the larger ones, and it is interesting that similar chromosome pairs differ in this respect - e.g. chromosome 17 appears to be lost more frequently than chromosome 18, and 21 more often than 22.

### iii. Effect of Culture Time on Detection of Hypodiploid Cells

In Fig. II, 3, 7 the figures for newborn babies and 14-year olds are pooled as are those for individuals of 76 and over, to decrease the fluctuations due to the low numbers of cells scored in these age groups. Below the age of 30 years, hypodiploid cells seem to be more frequent in 'late' cultures but in other age groups there is very little difference between 'early' and 'late' results (Table II, 3, 4). Generally, there are more hypodiploid cells in 'early' than 'late' cultures between the ages of 37 and 70 years, so this may be a reflection of an *in vivo* situation. A comparison between the lines is complicated by the fact that in individuals between the ages of 30 and 74 years larger numbers of cells were scored in 'late' than in 'early' cultures (Table II, 3, 1). However, the results do suggest that culture time is not markedly affecting the

proportions of A and B type cells with 45 chromosomes, and a comparison of the regression coefficients reveals no significant difference between 'early' and 'late' results.

iv. Proportion of Female Cells with Unstable Chromosome Aberrations, by Age

Cu cells show a highly significant increase with age (Table II, 3, 3a) and this is almost entirely due to the presence of cells with one or more 'X fragments', since other types of Cu cells show little change. Fig. II, 3, 8 and Appendix IIIc show the proportions of both categories of Cu cells with data from the 'early' and 'late' cultures presented separately. For Cu cells with 'X fragments', the influence of culture time is quite marked, with a much higher number of such cells in 'early' cultures; and it is notable that no cells of this sort are found in individuals below the age of 32 years. The graph showing the results from 'early' cultures shows an unexpected drop to zero at age 62.3 (Fig. II, 3, 8), but this point was obtained by scoring only 50 cells and is consequently of little significance. The scattergram (Fig. II, 3, 9) gives more convincing evidence for an age-related increase in Cu cells, in accordance with the regression results (Fig. II, 3, 10), than did the scattergram showing hypodiploidy data (Fig. II, 3, 4); but once again the marked individual variations in proportions of Cu cells are clearly illustrated. It is interesting that Cu cells of types other than those with 'X fragments' are slightly more frequent in 'late' rather than 'early' cultures, so that the 'X fragment' may be a reflection of an in vivo situation, while other Cu cells are more likely to

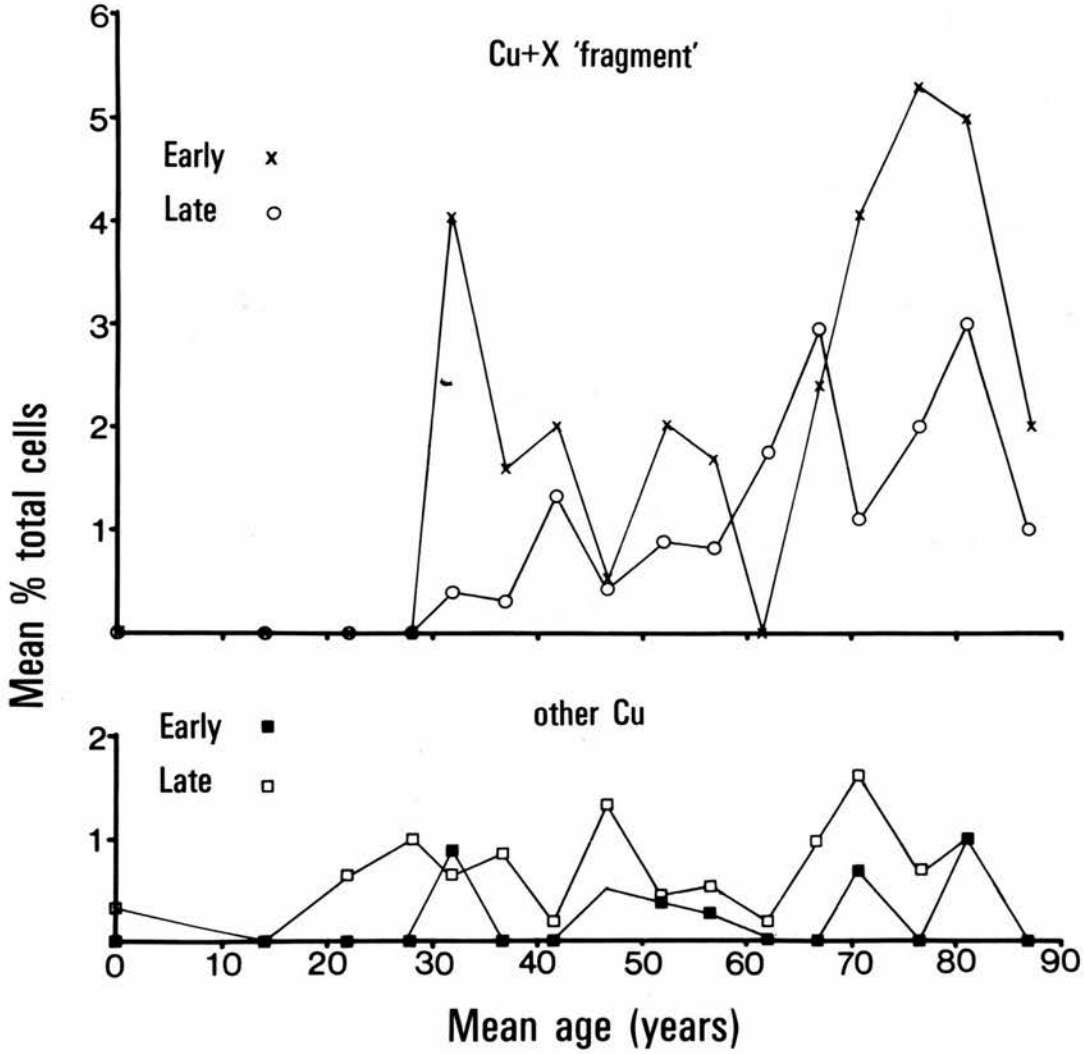
Cells with unstable chromosome aberrations

(Cu cells) in female sample.

Fig. II, 3, 8.

a. Cells containing one or more "fragments" of X.

b. True Cu cells, by age and culture time.


Cu+X 'fragment'
Early
Late
Mean % total cells
6
5
4
3
2
1
other Cu
Early
Late
2
1
0
10
20
30
40
50
60
70
80
90
Mean age (years)

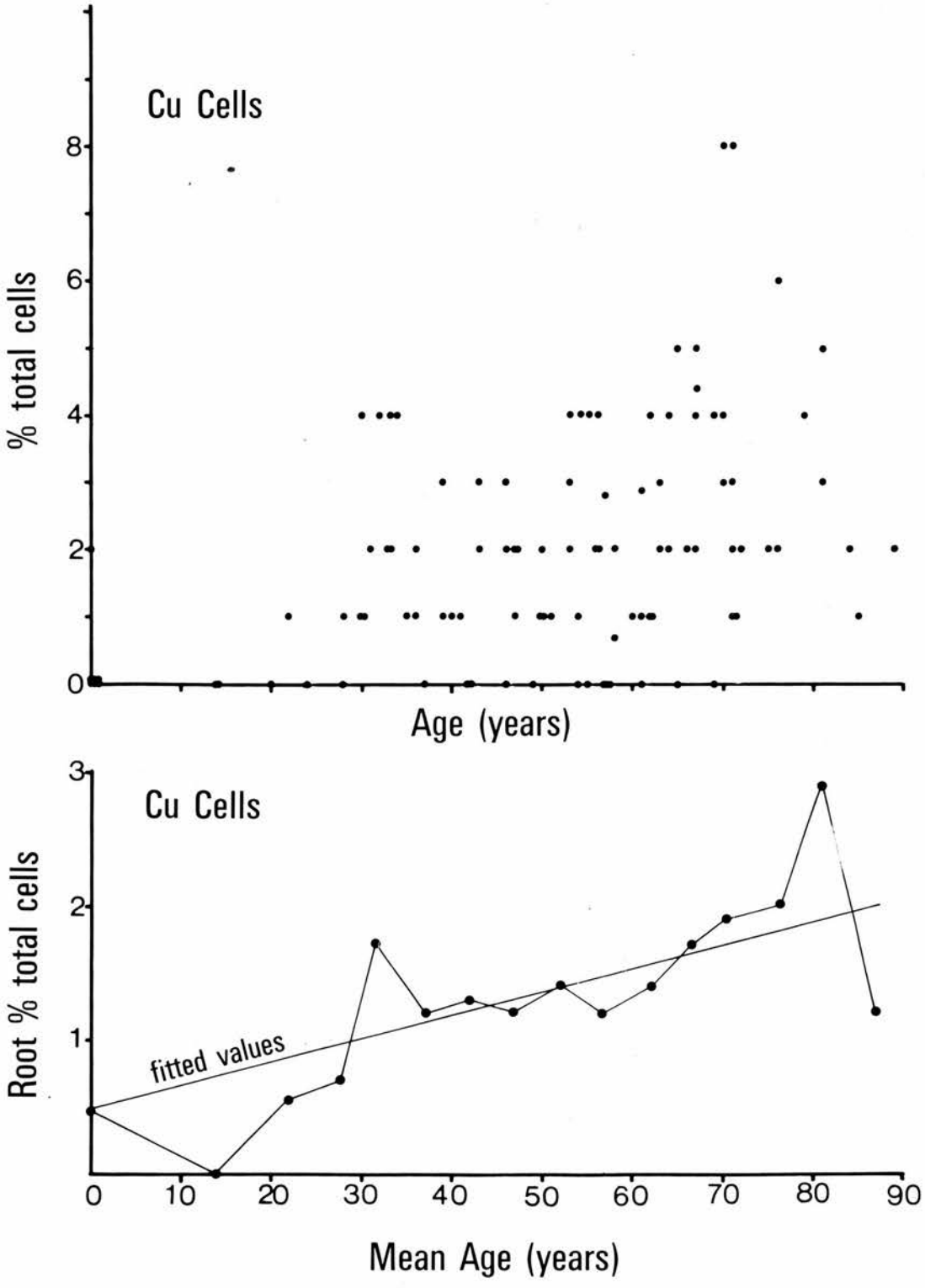
## Proportions of Cu cells in the female sample.

### Fig. II, 3, 9.

Scattergram of individual results, plotted against the age of each subject.

### Fig. II, 3, 10.

The square root of the mean percentage of Cu cells for each age group is plotted against the mean age for the group. The fitted line derived by weighted linear regression analysis is also shown.


Cu Cells
% total cells
0
2
4
6
8
Age (years)
Cu Cells
Root % total cells
fitted values
1
2
3
0
10
20
30
40
50
60
70
80
90
Mean Age (years)

arise in culture. No significant differences were found among these four regression coefficients (i.e., Cu cells with or without 'fragments' in early and late cultures).

Where only one 'X fragment' is present, 90% of the cells in 'early' cultures have the single 'fragment' in place of an X chromosome, and in the remaining cells the 'fragment' is present in addition to a complete complement. In late cultures 'additional fragments' are more common and occur in 17% of the cells with one 'X fragment' (Fig. II, 3, di). Multiple 'X fragments' are more common in 'late' cultures (Table II, 3, 5) (Fig. II, 3, dii).

v. The 'X Fragment'

The 'medium fragment' in female cells has a variable appearance (Figs. II, 3, c and II, 3, d) and can look exactly like a normal X chromosome but without any visible junction of the chromatids at the centromere, or may have the most frequently seen conformation, with sister chromatids lying straight and parallel with no constriction in the centromere regions. The 'fragment' may also be very much less contracted than the rest of the chromosomes, but usually retains the characteristic 'straight' appearance with 'chromatid apposition'. In cells with more than two 'fragments' it sometimes appears that two are paired fragments of similar appearance, while a third might have a completely different appearance, possibly being very much more contracted.

The G-bands of the 'X fragments' appeared to be those of a normal X chromosome and there is no reason to suspect

TABLE II 3 5.

Cu cells with "X-Fragments".

| Culture time | 1 "X fragment" | | 2 or more "X fragments" | Total cells |
|---|---|---|---|---|
| | replacement | additional | | |
| Early | 72% | 8% | 20% | 104 |
| Late | 52% | 11% | 37% | 83 |

Early;- 2 day culture

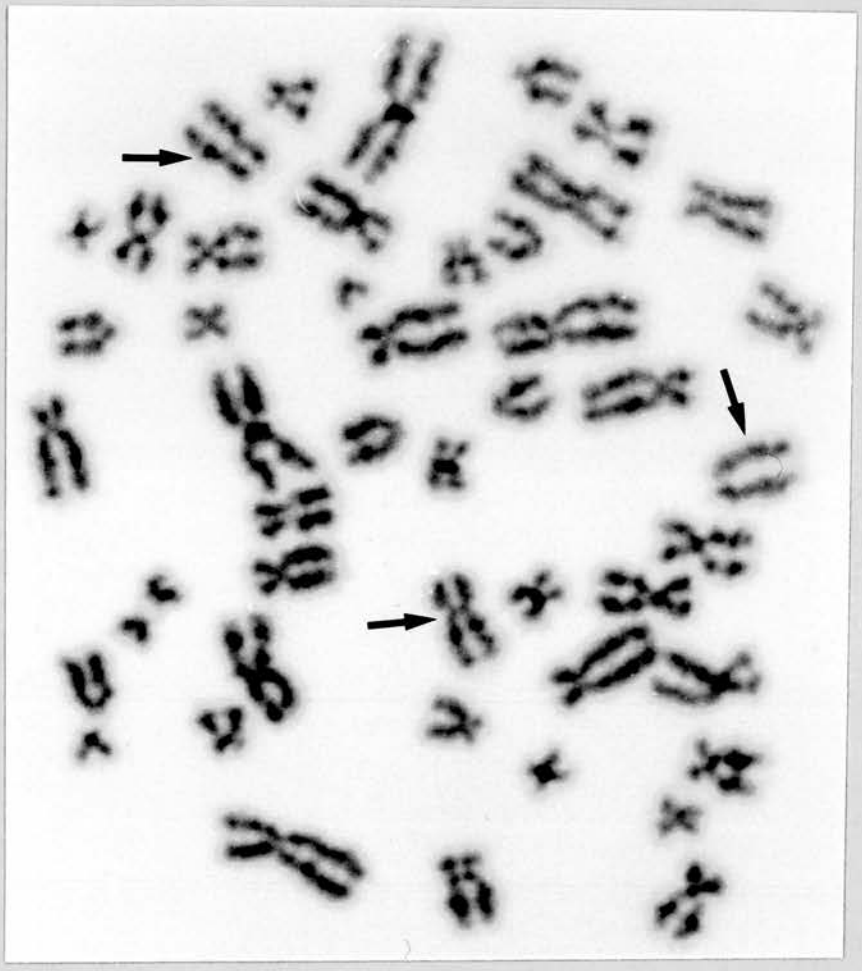
Late;- 3 day culture

Fig. II, 3, c i.

This cell from a normal female contains 47 objects. There is one apparently normal X chromosome, an X chromosome that is splitting at the centromere, and a "medium acentric fragment".

Fig. II, 3 c ii.

This collection of "X fragments" illustrates, with the photograph above, the variable appearance of the "fragment", which may show close chromatid apposition, or have two widely separated chromatids.



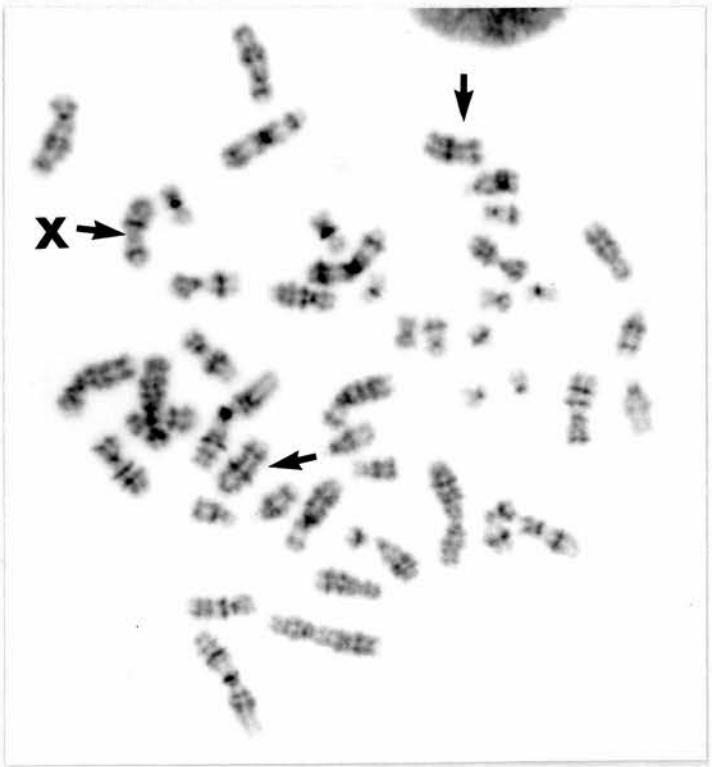
X 'FRAGMENTS'

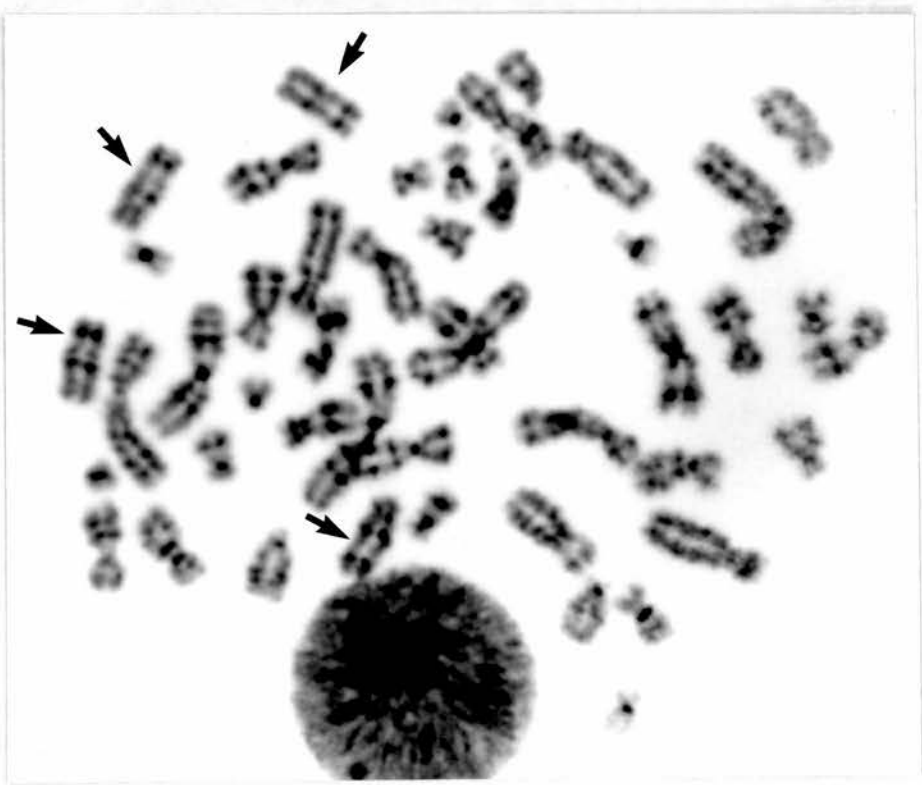
Fig. II, 3, d i.

This G-banded cell contains one replacement and one additional "X fragment", (arrows).

Fig. II, 3, d ii.

This cell was classified as having 44 chromosomes, and four "X fragments", (arrows). The cells was observed in a preparation from a 72 hour culture of blood from a female aged 64 years.


X



the loss of material that one might expect if this were truly an acentric fragment that had undergone an interstitial deletion to lose the centromere. Some G-banded cells with 'fragments' were destained after photographing or noting the position of the cells, and were treated by the BaOH C-banding technique, after which re-examination of the same cells showed the presence of a normal small C-band on the 'fragment', suggesting that the centromere has not been lost, (although the presence of centromeric heterochromatin does not guarantee the existence of a centromere). In conclusion, it appears that this 'fragment' is not a true acentric fragment but is a prematurely divided X chromosome with the characteristics of chromosomes that have divided at the centromere prior to cell division. This early chromatid separation may well be involved in the generation of hypo- and hyperdiploid cells by non-disjunction of the X chromosome (see below).

### vi. Late-Labelling Patterns of Cells with X Chromosome Aneuploidy and 'X Fragments'

The cultures treated with tritiated thymidine for the last few hours were G-banded and scored normally, before being destained and processed for autoradiography. BrdU was also used for late-labelling (see Part I, Chapters Two and Three) and slides were stained by the FPG method. In general, these were not fruitful experiments as the cells of interest rarely coincided with the cells showing clear late-labelling patterns and no clear results were obtained using the BrdU late-labelling technique, a technique that was only applied

to cells from a small number of individuals. However, a few cells were informative after autoradiography.

a. 47XXX Cells. One of these cells had two similar late-labelling chromosomes, so that the extra X chromosome is 'inactive', while a second cell had three differing X chromosomes, one unlabelled, one with the long arm only labelled and a third X that was labelled all over. The latter chromosome is the most retarded in DNA synthesis, since we know that the long arm of the X is more late-replicating still than the short arm (Gianelli, 1970; and Part I of this study). In this cell there are, therefore, two 'late X's', but they are not completely synchronous in their DNA synthesis patterns.

b. 48XXX + 15. Only one X chromosome in this cell was labelled. Since the 'active X' is also fairly late-replicating and its DNA synthesis period overlaps with that of the 'late X', it is necessary to exclude the possibility that the single labelled X in a 47XXX cell is the 'early X'. This can be done be examining the labelling 'status' of the other chromosomes in the cell, and in this particular cell it was possible to conclude that the single labelled X was in fact the 'late X'.

In conclusion, the additional X chromosome in a trisomic cell is not necessarily an 'inactive X'.

c. 45X

Of two cells with late labelling 'markers' such as the secondary constrictions of chromosomes 1 and 13, the remaining X chromosome was labelled in one and unlabelled in the second. A third cell with a labelling pattern suggesting it had taken up tritium at a slightly earlier time - i.e. the centromeric regions of chromosomes 3 and 16 were labelled - had a labelled X chromosome that was probably the late X, but cannot be unequivocally identified as such. These results do suggest that the late-replicating X is not exclusively the homologue that is lost from the 45X cells.

d. 46XX + 'X fragment'

One late-labelling X chromosome was seen in this cell but the 'fragment' was not labelled. None of the other cells with 'fragments' had taken up isotope. Again, this demonstrates that although the result is only for one cell, the 'fragments' need not always be formed by the late-replicating X chromosome.

vii. Repeat Cultures: Comparison of Results

Repeat cultures were analysed for 12 females aged 43 to 71 years, with periods of 11 days to 14 months in between the samples. The results (Table II, 3, 6) illustrate the problems of attempting to assess an expected proportion of abnormal cells for predictive purposes in a sampling system such as this, where a very small proportion of the total lymphocytes is selected. In one culture from any individual no Cu cells may be seen; while on a different occasion 5-6%

TABLE II 3 6.

Repeat cultures from 12 females.

| | Age | time between cults. | | percent abnormal cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Cu | | Cs | | hypodiploid | | hyperdiploid | |
| | | | | a | b | a | b | a | b | a | b |
| 1 | 43 | 14mo | E | | 4 | | 0 | | 4 | | 0 |
| | | | L | 3 | 4 | 1 | 2 | 4 | 6 | 0 | 0 |
| 2 | 56 | 13mo | E | | 4 | | 0 | | 12 | | 0 |
| | | | L | 2 | 4 | 0 | 0 | 8 | 8 | 1 | 0 |
| 3 | 57 | 13mo | E | | 2 | | 2 | | 4 | | 0 |
| | | | L | 0 | 0 | 0 | 0 | 6 | 0 | 2 | 2 |
| 4 | 61 | 9mo | E | | 10 | | 0 | | 16 | | 2 |
| | | | L | 2.9 | 10 | 0 | 2 | 5.9 | 14 | 1.5 | 2 |
| 5 | 62 | 10mo | E | | 0 | | 0 | | 8 | | 0 |
| | | | L | 4 | 6 | 0 | 0 | 9 | 10 | 0 | 0 |
| 6 | 65 | 10mo | E | | 2 | | 0 | | 2 | | 0 |
| | | | L | 5 | 6 | 0 | 0 | 8 | 4 | 8 | 2 |
| 7 | 66 | 13mo | E | | 6 | | 0 | | 8 | | 0 |
| | | | L | 2 | 0 | 0 | 0 | 5 | 8 | 5 | 2 |
| 8 | 67 | 12mo | E | | 2 | | 2 | | 6 | | 0 |
| | | | L | 2 | 2 | 0 | 0 | 7 | 2 | 2 | 0 |
| 9 | 67 | 11d | E | 4 | 14 | 2 | 0 | 14 | 6 | 0 | 0 |
| | | | L | 6 | 0 | 2 | 0 | 4 | 0 | 4 | 0 |
| 10 | 71 | 17d | E | 10 | | 2 | | 16 | | 0 | |
| | | | L | 6 | 0 | 2 | 0 | 6 | 4 | 2 | 0 |
| 11 | 71 | 11d | E | 4 | 9.7 | 0 | 3.2 | 10 | 9.7 | 4 | 3.2 |
| | | | L | 2 | 2 | 0 | 2 | 12 | 8 | 0 | 2 |
| 12 | 71 | 11d | E | 2 | 0 | 0 | | 4 | | 2 | |
| | | | L | 0 | | 0 | 0 | 8 | 6 | 4 | 0 |

a. first sample

b. second sample

E early (2 day culture)

L late (3 dqy culture)

of the cells may have chromosome aberrations. In two samples cultured only 11 days apart (individual number 9) the proportion of 45X + 'X fragment' cells increased from 4% to 12%. In this individual it was also notable that the frequency of 14% Cu cells in the 'early' culture was reduced to zero by the 72 hour culture..

Table II, 3, 6 also illustrates the individual variations even among females of the same age; for example, individuals 4 and 5 have 10% and 0% Cu cells respectively in 'early' cultures, and individual 10 has 10% Cu and 16% hypodiploid cells compared with individual 12 who has only 2% Cu and 4% hypodiploid cells.

## D. ANALYSIS OF THE DATA FROM THE MALE SAMPLE

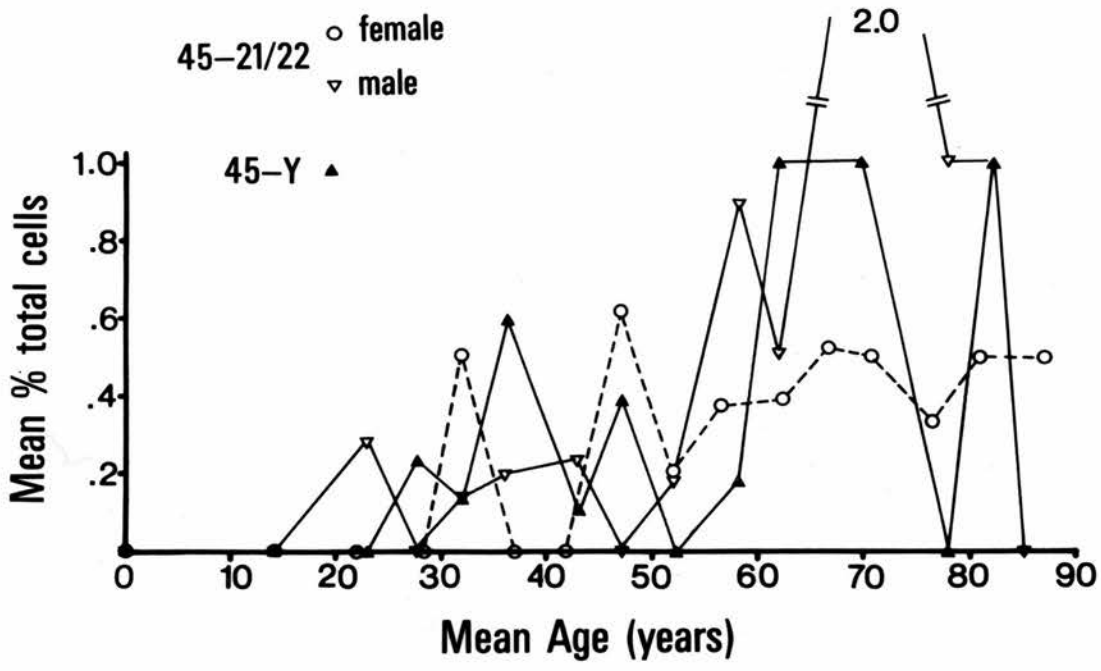
### i. Hypodiploid Cells: Identification of Lost Chromosomes and Sex Chromosome Aneuploidy

The data are shown in detail in Appendices IIId and IIIe. The striking feature of the male results shown in Table II,3,4 is the loss of the Y chromosome, but it is noteworthy that while 14/90 cells were 45X-Y, a further 15 of the 90 have lost chromosome 21 or 22, and of these chromosome 21 is much more commonly lost. This loss of G-group chromosomes is important as it illustrates that we cannot assume that cells identified in orcein-stined preparations that have lost a small acrocentric are all 45-Y. It is also interesting that no cells that had gained a Y chromosome were seen, although the female X chromosome aneuploidy includes a high proportion of 47XXX cells among the hyperdiploid cells. Fig. II, 3, 11 shows the frequency of the cells that have lost the Y or G-group chromosomes with age, in both males and females. The number of males in any age group who show any Y loss does increase slightly with age after 44 years, but the total number of cells missing a Y does not seem to depend on age; only 1/100 cells had lost a Y chromosome in all the individuals examined except for one, aged 35 (3/100 cells) and one aged 63 years (2/100 cells). The numbers altogether are very low, and always below 1% of the cells scored, and the increase in 45-Y cells is not significant (Table II, 3, 4b), but the total numbers of cells examined overall are too small to draw any firm conclusions.

Fig. II, 3, 11.

Cells missing small acrocentric chromosomes.

The proportions of cell missing a chromosome number 21 or 22 are compared for males and females. The numbers of cells missing a Y chromosome in males are also shown.


45–21/22
female
male
45–Y
2.0
1.0
.8
.6
.4
.2
Mean % total cells
0
10
20
30
40
50
60
70
80
90
Mean Age (years)

Unlike the situation in females, hyperdiploid cells do not increase significantly in males, although hypodiploid cells do. It is clear that the loss of X chromosomes seen in females is absent in males, and the total frequency of cells that have lost a medium-sized chromosome in males, 29.6%, is close to the frequency of 32.6% expected if loss is random and depends on numbers of chromosomes only. The most frequently lost (6-12) group chromosome in males is number 10. A comparison between regression results for male hypodiploid cells and for female hypodiploid cells excluding the large numbers of 45X cells, shows there is no significant difference ($t = 0.86$), so that the difference between male and female rates of hypodiploidy may largely be accounted for by the female sex chromosome loss. The loss of chromosomes 21 and 22 seems to be more marked in male than female cells, at ages over 55 years (Fig. II, 3, 11; Appendix III), although these results are based on rather small numbers of cells.

As in female cells, chromosomes of the same size group are not lost with equal frequency, but while chromosome 21 was more commonly lost than 22 in both males and females, the male pattern for E-group loss is the reverse of the female situation, with chromosome 18 more frequently lost than 17, and this is probably an indication that conclusions on the comparative frequencies of chromosome loss should not be drawn from results on such small numbers of cells.

ii. Proportions of Male Cells with Unstable Chromosome Aberrations, by Age

A 'medium fragment' with G-bands of an X chromosome appears occasionally in male cells also, and was seen altogether in eight cells from seven individuals aged 35 to 59 years. In six cells the 'fragment' was replacing an X chromosome, and in one cell it was additional. The remaining cell had twin 'X fragments' in addition to a normal complement. This is further proof that the 'X fragment' is not necessarily formed by the late-replicating X chromosome. 'Medium fragments' were seen in two other individuals, but these were prematurely divided chromosomes 7, 12 and 8 in cells where the remaining chromosomes still appeared 'tightly bound' at the centromeres.

In two cells, small fragments were seen that looked like the Y chromosome and were replacing the Y. The Y often lies in a characteristic manner, with long arms parallel or in 'chromatid apposition', but in these two cells the sister chromatids were quite clearly separated at the centromeres.

## E. CELLS WITH ABERRATIONS IN MALES AND FEMALES

### i. Chromosome Aberrations

Cells with stable chromosome aberrations (Cs cells) were rare and did not show much age-related change in males. In females, Cs cells did show an overall increase but this was only significant in 'early' or pooled results, and not in 'late' culture results alone. Overall, the proportion of Cs cells remained well below 1% of the total cells (Apendix III, c and e).

Cu cells, that is to say cells with true unstable chromosome aberrations such as dicentrics and rings and not including the cells with 'X fragments', did not increase (Fig. II, 3, 8; Table II, 3, 3; Appendix III) and were more common in late cultures. Details of the aberrations and the chromosomes involved are shown in Table II, 3, 7. There are not enough cells to remark on the involvement in rearrangements of any particular chromosome although it is interesting that the (6-12) group is commonly involved, and dicentrics involving the X chromosome occurred twice in female cells, although there is evidence for non-participation of this chromosome in exchanges induced by irradiation in $G_1$ (see, for example, Seabright, 1973b). However, the recent studies by Buckton (1976) of $G_0$ irradiation and by Savage et al (1973) using $G_2$ irradiation, show no lack of X chromosome involvement in aberrations. The overall frequency of dicentrics (about 1 in 1100 cells) is very similar in males and in females (Table II, 3, 7) and only

TABLE II 3 7

i. Chromosomes involved in rings and dicentrics.

| Individuals | | Early | | | Late | | |
|---|---|---|---|---|---|---|---|
| sex | age | chromosomes dicentrics | involved rings | frag-ment* | chromosomes dicentrics | involved rings | frag-ment* |
| Males | 23 | 3-9 | | - | | | |
| | 34 | | | | 13-20 | | |
| | 46 | 12-14 | | - | | | |
| | 78 | 1-12 | | + | | | |
| | 80 | 1-1 | | + | | | |
| Females | 43 | | D | | | | |
| | 43 | | | | 1-C | | - |
| | 46 | | | | | 2 | - |
| | 47 | | | | 9-10 | | - |
| | 51 | | | | ? | | - |
| | 53 | 2-7 | | + | | | |
| | 61 | | | | 7-X | | + |
| | 63 | | | | 10-X | | + |
| | 70 | | | | 12-17 | | - |
| | 71 | 5-7 | | - | | | |
| | 71 | | | | 11-12 | | + |

ii. Frequency of dicentrics.

| | Early | | Late | | Total |
|---|---|---|---|---|---|
| | dicentrics | cells | dicentrics | cells | |
| Males | 4 | 2380 | 1 | 3622 | 1 in 1200 |
| Females | 2 | 3521 | 7 | 5906 | 1 im 1047 |
| TOTAL | 6 | 5901 | 8 | 9528 | 1 in 1102 |

* fragment associated with dicentric or ring.

two rings were seen altogether, so that these occur with a very low frequency (1 in 4700 cells). None of the patients in whose cells these aberrations were noted had had any known exposure to radiation other than routine diagnostic X-rays, nor were any of these individuals taking any form of medication.

ii. Chromatid Aberrations

'B' cells include cells with chromatid gaps, breaks and interchanges, and in G-banded preparations the chromatid gaps detected commonly in orcein-stained cells are not easily visualised because of their resemblance to pale G-bands. The frequency of chromatid aberrations in general did not alter with age, and was higher in 'early' cultures (0.8 to 6% of the cells) compared with 'late' cultures (0.3 to 2% of the cells) (Appendix III). The overall frequency of chromatid aberrations in a total of 15,429 cells was 1 in 71 cells or 0.041 per cell. Of the 217 aberrations:

12% were isochromatid gaps (0.0017/cell)
59% were chromatid gaps (0.0083/cell)
27% were chromatid breaks (0.0038/cell)
and 2% were chromatid interchanges (0.0003/cell)

Of the five chromatid interchanges found, two were in cells from one individual, and each was a homologous interchange between the C-band regions of chromosome 9. Chromosomes of this individual had a large secondary constriction visible by G-banding and confirmed by BaOH C-banding.

Using the information obtained by the Clinical section of this Unit, I assigned the patients to two groups, one of which included all individuals known to be taking drugs of any description. The numbers of cells with chromatid aberrations in this group (46/3240 cells, or 1.35%) did not differ significantly from the proportion of 'B' cells from people who were not under medication (89/7081 cells, or 1.26%.

# CHAPTER FOUR

## DISCUSSION

The results of this survey confirm that aneuploidy increases with age and that there is a significant sex difference in the nature of the aneuploid changes. In females there are significant increases in both hypodiploid and hyperdiploid cells, and also in cells with stable or unstable chromosome aberrations, while in males only hypodiploid cells are significantly increased. The use of C-banding has allowed me to establish that a large proportion of aneuploidy in female cells is due to loss or gain of an X chromosome, and the increase in 'Cu' cells is mainly due to the appearance of cells with one or more prematurely divided X chromosomes, replacing, or in addition to, the normal X chromosomes. In both males and females, cells lacking a small acrocentric chromosome also make a large contribution to the total of hypodiploid cells, and one or other of the G-group autosomes (chromosomes 21 and 22) is lost as frequently as the Y chromosome in males, so that it is clearly erroneous to assume that orcein-stained cells from males, which lack a small acrocentric chromosome, are all (45X, -Y).

### i. Incidence of Chromosome Aberrations

The background numbers of chromosome aberrations are of interest because of the possible role of mutagen exposure in inducing such aberrations in the cells of exposed individuals.

a. Chromosome Aberrations. The levels of Cs cells detected here are slightly lower than those reported in the earlier Edinburgh study (Court Brown et al., 1966; 1967) and I find an increase in Cs cells with age in females but not in males, a result that is the opposite to the findings of the 1966 study. However, the low numbers of Cs cells overall allow no very marked trend to be ascribed to the proportions of these cells with age, and it is probable that any age-related changes in Cs cells could be due to accumulating exposure to diagnostic X-rays, for example, rather than a true effect of age per se.

b. Chromatid Aberrations. The frequency of chromatid aberrations ('B' cells) detected in orcein-stained cells has been variously assessed, but the results of this study correspond closely to those quoted by Evans (1970) from examinations of less than 5000 cells from 50 hour peripheral blood leukocyte cultures. The results of Court Brown (1967) are probably more comparable to mine, as they include scores on 12,420 cells from both 48 and 72 hour cultures, and the numbers of chromatid breaks in my results compare favourably with the 1967 study, although my scores for chromatid- and isochromatid-gaps are lower, possibly due to my use of G-banded preparations, where these aberrations are more difficult to detect. Court Brown et al (1966) found a slight decrease in B cells in females but B cells in my study show no age-related changes in either sex. The results of the present work thus show a slight overall increase in the incidence of 'chromosome breaks', unlike the data from the study by Sandberg et al (1967) on a New York State population

where the number of 'breaks' was found not to be influenced by age or sex.

ii. Aneuploidy

a. Effect of Culture Length.

In comparing the numbers of hypodiploid cells found in two- and three-day cultures, Court Brown et al (1966) reported that higher proportions were found in the 'late' cultures, but I have not found a consistent difference (Fig. II, 3, 7; Appendix IIIa). The pooled results from both culture times in my data seem to give a sufficiently accurate picture of the changing proportions of these cells with age, and the effects of culture time are fairly insignificant in comparison with the wide variations among individual scores. In my study hyperdiploid cells were generally slightly more frequent in late cultures from males, but culture length had no consistent effect on the proportions of hyperdiploid cells in females.

b. Nature of the Aneuploid Changes.

The increase in hypodiploidy in females described by Jacobs et al (1963) was shown to fit a cubic curve, with a faster rate of increase between the ages of 45 and 64 years than at other stages. A similar pattern was found in the study of the population of the island of Tristan da Cunha (Hamerton et al., 1965), while the New York study (Sandberg et al., 1967) showed a high proportion of hypodiploid cells in females over 60 years, but no steady increase below this age. My results, illustrated in Figs. II, 3, 2, 4 and 5,

show no clear increase in hypodiploidy between the ages of 30 and 60 years, and possibly a much faster increase after this, so that in general the results are more consistent with those of Sandberg's New York study than with the studies of Jacobs et al (1963) and Hamerton et al (1967), both of which described a rapid increase in aneuploidy during middle age. On the other hand, the results of the present study and from Court Brown et al (1966), when plotted as a scattergram of individual results, show a stronger hypodiploidy-age relationship than do the New York data (Sandberg et al, 1967) where many individuals of below 30 years had large numbers of hypodiploid cells (up to 10% of the diploid cell count, with several scores that were higher still). This discrepancy is not easy to explain, as any differences between studies in methods of cell selection and scoring should affect the results to the same extent at all ages.

In males, the pattern of hypodiploidy shows a fairly even level between about 20 and 55 years, followed by a faster increase and an apparent drop in very old individuals. The decrease in hypodiploidy seen in very old people here is almost certainly due to the small sample size, and the largest study, on 189 people over 65 years of age (Jacobs et al., 1964) showed a continuing rise in hypodiploidy in these individuals from about 8% of modal cells in the 65-69 year age group to about 15% in people over 80 years old.

Court Brown et al (1966) stated that in females aneuploidy began to increase markedly between 55 and 65 years, and that in males the changes appeared later and were less dramatic.

In my study the proportions of hypo- and hyperdiploid cells are very similar to those reported by the Edinburgh group (Court Brown et al., 1966), but I have found that the increase in hypodiploid cells and in cells with chromosome and chromatid aberrations begins just as early in males as in females, although the males in general had fewer hypodiploid cells overall. This latter difference was shown to be accounted for by the preponderance of 45X cells in females. The increase in hyperdiploid cells seems to follow a steady pattern with no good evidence for the steeper increase after 45-54, described by Court Brown et al (1966).

It is clear that individual variations are very large, and the results of repeat cultures from single individuals (Table II, 3, 6) illustrated that large numbers of individuals would have to be sampled to give a fair assessment of the mean frequency of hypodiploidy in any age group. It is also apparent from the scattergrams that a strong relationship found by regression analyses on grouped data may be less convincing when the range of individual results is presented.

Sandberg et al (1967) stated that only about 7% of the hypodiploidy observed is associated with age and the rest with "unknown factors". The percentage of the variance accounted for by a regression on age for hypodiploid cells in my data was 43.5% for females, and 37.9% for males, so that we must still attribute over half the variation to influence other than age. In the results from the computer regression analysis, the strongest age relationship was for

female Cu cells, and even for those cells with 'X fragments' the percentage of the variance due to age was only 63.7%, so that other factors must be affecting the proportions of these cells.

Culture artefacts may well lead to a certain amount of the observed aneuploidy, particularly since broken cells may be counted as hypodiploid cells despite careful selection. Hyperdiploid cells may also include chromosomes from other cells, and although these may sometimes be recognised due to their different degree of condensation, or their position at the edge of the cell, there may also be some cells where foreign chromosomes are not recognised. In the 'harlequin'-stained cells described in Part I, cells were sometimes seen where chromosomes that were recognisable by their staining pattern to originate from M1 or M3 cells, were lying well inside the boundaries of an M2 nucleus, and since they were of the same degree of contraction they would not have been recognised by conventional staining techniques.

In considering age effects it seems logical to take as a baseline level of aneuploidy due to culture artefacts, the frequency of aneuploid cells found in samples from new-born babies. It is clear that there is an age-related increase in aneuploidy over this background level, and that the involvement of particular chromosomes in aneuploidy is non-random.

iii. Non-Random Chromosome Loss

The 1966 study (Court Brown et al) presented evidence that all the increase in aneuploidy in females was due to cells lacking a C-group chromosome, but I found that cells missing chromosomes other than the C-group (6-12 + X) also increased significantly (Table II, 3, 3a; Fig. II, 3, 6) although the proportions were lower (a maximum of 2.15% cells missing non C-group chromosomes compared with up to 10% 45X cells); and the increase appeared mainly after the age of 65. The 1966 Edinburgh study also showed that in females under 55 years, the numbers of cells with 45 chromosomes varied randomly from 0.6 to 1.9%, but I find a variation from 0 to 3.5%, followed by a rise to about 5% to 9% at ages over 65 (Fig. II, 3, 6). In males the increase in hypodiploidy is mainly due to the cells that have lost a small acrocentric - i.e. 21, 22 or the Y chromosome - and the total number of cells in this study that had lost a Y was equivalent to the total number of cells missing a 21 or 22, so that there is certainly a disproportionate loss of the Y, although the overall number of male 45X cells is very small. The number of individuals showing Y loss is possibly related to age, but the degree of Y loss is not, in accordance with the results of Pierre and Hoagland (1972) who studied bone marrow cells.

The distribution of chromosome loss from each size group in this work is similar to that published in 1966 (Court Brown et al), and the use of G-banding has revealed that there may be differences in the frequency of loss among members of any size group, for instance chromosome 17 may be more frequently

lost than 18. Since this was not a consistent result in both the total male and total female data, it may simply be an artefact due to the small numbers of cells involved, but this sort of result could indicate a selection process whereby cells that have lost certain chromosomes are more likely to survive than others. If this were true, we might expect that cells that lost an inactive X chromosome might be more fit for survival than cells missing the active X chromosome that provides the coding information for essential enzymes required for cell metabolism. The auto-radiographic studies described in this work suggest, however, that it is not exclusively the inactive X chromosome that is involved in aneuploidy, although the numbers of informative cells are very small.

a. Mechanism of Sex Chromosome Aneuploidy. The 'X fragment' seems to be a chromosome that has divided prematurely at the centromere and this process may make an important contribution to X chromosome aneuploidy, since if the centromere is abnormal it may not behave normally during cell division, thus increasing the chances of non-disjunction. A cell containing a normal X chromosome and a replacement fragment might, therefore, give rise to one daughter cell with only one X chromosome and a second cell with one X chromosome and two 'X fragments' derived from the chromatids of the original 'fragment'. It is also possible that the two chromatids of a 'fragment' may each give rise on replication to a normal X chromosome rather than one with an abnormal centromere that divides prematurely; that is to say, the centromere behaviour may not be consistently

inefficient over successive cell cycles. This mechanism would give rise to 47XXX cells. If a fragment does tend to give rise to two normal X chromosomes rather than to two fragments, we would expect the proportions of 47XXX cells to be greater than the numbers of cells of the type 45X + X fragment. This is in fact the case, suggesting that,

a. the cells with the 'fragment' may be lost due to difficulties at mitosis; or

b. chromatids from the 'fragment' are more likely to give rise to normal X chromosomes than to another 'X fragment' at the subsequent metaphase; or

c. there are other factors involved in the generation of 47XXX cells.

The involvement of the 'X fragment' in X chromosome non-disjunction has also been recently suggested by Fitzgerald (1975) who also demonstrated the presence of a C-band and concluded that the 'medium fragment' arises due to premature centromere division.

It is now clear that the occurrence of 45X cells in cultures from older females is an age-related phenomenon and an individual of 60 years old with an X chromosome missing in perhaps 8% of the cells scored is unlikely to be a constitutional 'Turner mosaic'. However, in one 33 year old female in the present sample, 4% of the cells from a three-day culture had a 45X karyotype. This might have been interpreted as evidence for a 45X (or 'Turner') cell-line in the absence of any other information about this patient, but for

the occurrence in the two-day culture of several cells (8%) that had the karyotype 45X + 'X fragment'. It is likely that loss of the 'X fragment' from cells of this type was giving rise to the 45X 'cell-line' in the 'late' culture. On several other occasions a high proportion of 45X + 'X fragment' cells in early cultures was associated with a considerable number of 45X cells detected in late cultures. This not only lends support to the idea that premature centromere division is a mechanism of X chromosome loss, but illustrates the caution necessary in interpretation of results of analyses of 'late' culture cells.

The single cell with an 'X fragment' examined by autoradiography in this study had a late-labelled X chromosome and an unlabelled X fragment, and since the 'X fragment' also appeared occasionally in male cells premature separation at the centromere is obviously not an exclusive feature of the inactive X chromosome.

b. Reasons for Non-Random Chromosome Loss.

1. Survival. In discussing the non-random nature of chromosome loss there are several possibilities to consider, the first being the assumption that chromosome loss is in fact random but that only certain types of cells survive. We do not have enough information to show whether the inactive X is preferentially lost from female cells, and if this is not the case it is hard to see why 45X cells should be particularly fit for survival as the X carries many important genes, including that for $G_6PD$, an enzyme that is essential

for survival of any cell type. One could suggest that there might be compensatory reactivation of an X after the loss of an active X chromosome, but the bulk of the evidence is that X inactivation is stable (see, for example, Migeon, 1972; Cattanach, 1975), although there has been some suggestion of a localised derepression of certain genes on the X in human-mouse hybrid cells (Kahan and Demars, 1975; Pearson, personal communication) and Rao and Janawhar (1975) have also reported reactivation of an inactive X in 13% of bone marrow cells of the bandicoot. In studies in tissue culture systems it is important to avoid confusion arising from the appearance of certain enzymes as a consequence of mycoplasma contamination (Stanbridge et al., 1975).

The loss of the Y chromosome from male cells happens much less frequently and in much older individuals than does X chromosome aneuploidy. This is interesting since the genes known to be carried on the Y chromosome, such as those for testicular differentiation and maturation (reviewed by Siebers et al, 1973) and for the male histocompatibility antigen, H-Y antigen (Koo et al., 1976) are unlikely to affect directly the ability of a lymphocyte or marrow cell to survive. Thus, if the idea that all chromosomes are increasingly likely to be lost with age, and the numbers of surviving cells missing particular chromosomes depends on the contribution of that chromosome to the necessary function of the cell, we would expect many more 45-Y cells in males and the pattern of loss would be more like that of the X in females.

2. Order in the Nucleus. A second possibility is that the pattern of loss reflects some internal order of the chromosomes in the nucleus, implying that the most frequently lost chromosomes such as the small acrocentrics normally lie near the periphery of the nucleus. It is also possible that the inactive X might be lost simply because it is so late replicating that it might not condense in time to undergo normal mitosis.

3. Centromere Separation. The process of centromere division may be important in the generation of aneuploid cells, and from studies of centromere separation of both human (Vig and Wodnicki, 1974) and Chinese hamster chromosomes (Vig and Millenburger, 1976) it was concluded that this process follows a strictly ordered sequence. These authors suggested that the time of centromere division might be related to the centric heterochromatin content in such a way that the chromosomes having the largest amounts of this material would be the last to divide. In human cells the G- and D-group chromosomes separated last of all, and in the Chinese hamster cells the heterochromatic X and Y chromosomes were latest. If this effect applied to facultative heterochromatin, we might expect the inactive X chromosome to be less likely to divide prematurely than the active X. It is interesting that although Vig stated (Vig and Wodnicki, 1974) that in human cells the Y chromosome was one of the earliest to divide, I have only twice seen an apparent 'Y fragment' and 47YY cells are rare, while 45X cells are much less common in males than in females. Obviously we must distinguish between facultative and constitutive heterochromatin in

considering the effects of heterochromatin content on centromere separation, and it does seem more likely that centromere behaviour should be influenced by constitutive centromeric heterochromatin rather than by the facultatively heterochromatic state associated with inactivation of the whole X chromosome. According to Vig and Wodnicki (1974) the acrocentric chromosomes bearing the nucleolar organisers are the last to separate, yet in the age-associated increase in chromosome loss being considered here chromosome 21, a small acrocentric, is commonly lost, so that the order of centromere division does not bear a simple relationship to the likelihood of chromosome loss.

It is possible that premature centromere division, position in the nucleus and cell selection are all operating together, along with some other mechanisms that we do not yet recognise, to produce hypodiploidy.

iv Significance of Aneuploidy in Lymphocytes.

Having detected some age-related changes in chromosome behaviour, we must ask what the significance of these changes may be and whether they are relevant to the theory of somatic mutation or other hypotheses on the mechanisms of ageing. The evidence for an increase in chromosome breakage is not conclusive and any increase is fairly slight, but hypodiploidy does show a definite increase. This is not strictly evidence for mutation, though it could reflect a change in the processes that govern normal cell or chromosome division or in some less specific factor such as membrane fragility.

1. Relationship of Results to Chromosome Behaviour In Vivo. It is important to consider whether chromosome observations made on cultured lymphocytes are a true reflection of an in vivo situation. The aneuploidy seen in direct marrow preparations (Pierre and Hoagland, 1972) suggests that the age-related changes seen in blood are probably 'real', and also that in lymphocyte studies we underestimate the actual amount of abnormality arising in a rapidly dividing tissue like marrow, since blood lymphocytes do not normally divide.

Thus, although the relevance of lymphocyte studies to other tissues is always a controversial point it is more likely to involve differences of degree rather than any basic property. On the other hand, even within the lymphocyte group there are subpopulations with differing characteristics (see page 159; and Beek and Obe, 1974) such as sensitivity to radiation (Hand et al, 1974; Santos Mello et al., 1974; Janeway, 1975) and drugs (Abdou et al., 1973). In general, however, results in lymphocytes do apparently parallel those in marrow and fibroblasts, but there is no good evidence that observations on any of these tissues are relevant to the situation in meiotic cells although the increase in mitotic non-disjunction may be evidence for a defect in the processes governing cell division in general.

2. Relationship to Meiotic Non-Disjunction. Although we cannot extrapolate results from lymphocytes to meiocytes, the increase in aneuploidy in female lymphocytes is interesting

in the light of the increase in meiotic non-disjunction in females of over about 35 years, leading to the higher incidence of Down's syndrome cases (Penrose and Smith, 1966) and possibly to an increased risk of sex chromosome abnormalities, although the influence of parental age on incidence of sex chromosome aneuploidies is not yet clear. The information collected by Court Brown et al (1969) for sex chromosomally abnormal individuals identified in such situations as mental subnormality institutions or endocrinology clinics suggested that the risk of producing a 47XXY, 47XXX, or doubly aneuploid individual did increase with maternal age but that 47XXY or 45X individuals were not born more frequently to older mothers. A recent analysis of the data from this laboratory (A. Carothers, personal communication) confirms the increased incidence of XXY and XXX offspring in females over 35 years. The data from several surveys of newborn babies are conflicting. The results published by Hamerton et al (1975) were interpreted as evidence for a maternal age effect on numbers of sex chromosomally aneuploid children and Lubs and Ruddle (1970) found an age effect on the frequency of major chromosome abnormalities in general, while this was not confirmed by Friedrich and Nielsen (1973).

It is possible that any increased risk of chromosomal anomalies might be due not to an increase in likelihood of meiotic errors, but to a decrease in the efficiency of selection against abnormal foetuses by the maternal systems. Also, the mitotic non-disjunction seen in the lymphocytes becomes obvious in women who are past child bearing age

rather than at the age where an increase in abnormal offspring becomes obvious.

3. Aneuploidy and Malignancy. Sex chromosome loss may also be associated in some complex manner with malignancy, and there is evidence from marrow studies that in males with leukaemia the 45X cells and cells with the $Ph^1$ chromosome but no Y chromosome are less likely to contain other chromosome aberrations (reviewed by Sakurai and Sandberg, 1976), although the relevance of these observations is not clear. It is possible that the cancer processes prevent the normal age associated loss of the Y, rather in the manner that cells transformed in vitro do not show normal senescence (Pierre and Hoagland, 1973). Loss of the X chromosome has also been reported in marrow cells in acute leukaemia (Rowley, 1974) and of both the X and Y in human tumours such as meningiomas (Zankl et al., 1975) where it seemed that in some cases the inactive X was preferentially lost since the frequency of Barr bodies in interphase cells was reduced.

The available information on chromosomes and cancer does not show a disproportionate involvement of sex chromosomes only, however. The association of specific chromosomes or chromosome rearrangements with various malignant or non-malignant myeloproliferative diseases has been described in Part I (Chapter Five, A, iv) and reviewed by Rowley (1975; 1976). The common appearance of clones of cells containing a 'marker' chromosome 14 in Ataxia Telangiectasia lymphocytes or in marrow cells from patients with certain lymphomas is interesting,

and in haematological disorders, especially in Acute Myeloid Leukaemia (AML; Rowley, 1975) marrow cells are often found that are trisomic for C-group chromosomes, in particular chromosome 8 and sometimes 9. Marrow cells may also be monosomic for all or part of chromosome 7. The involvement of chromosome 8 is important as it now seems that one class of marker chromosome 14 is the result of a translocation between chromosomes 8 and 14 (Zech et al., 1976) and in AML an 8:21 translocation also occurs (Rowley and Potter, 1976).

In the present study, in the few trisomic cells besides 47XXX cells, chromosomes 8, 9, 6, 7 and 10 were involved, but not chromosomes 11 and 12 (Table II, 3, 4). In general the pattern of aneuploidy in these normal lymphocytes does not parallel that in abnormal bone marrow, as we might expect since the selection factors operating in marrow and blood are clearly very different and preparations from marrow are also made directly, in contrast to the two to three day culture period necessary for examination of blood lymphocyte chromosomes.

4. Aneuploidy and Other Physiological Changes. Exaggerations of the age-associated chromosome loss have been reported in association with senile dementia (Nielsen, 1968), raising once again the question of whether aneuploidy is the cause or the result of the ageing process, although studies in this laboratory did not confirm that demented patients had higher levels of aneuploidy than sex- and age-

matched controls (Jacobs, Buckton et al., unpublished data). The sex differences seen in the study of ageing by Jacobs et al (1963) and the timing of the aneuploidy increases were thought to indicate a possible correlation with such processes as hormonal changes in women of 40 to 50 years, associated with the menopause. Many other factors are altered in people as they age and thyroid activity may be important: an increase in the occurrence of Down's syndrome has been described in the offspring of women with thyroid disease (Fialkow, 1970), although it is possible that some other factor causes a more general distrubance that predisposes to both thyroid autoimmunity and to chromosome anomalies. Again, we cannot say that the birth of the chromosomally abnormal child is due to increased non-disjunction rather than to lack of the normal rejection mechanisms for an abnormal foetus.

There is also evidence for association of other physiological factors with chromosome aberrations, for example the effects of high blood pressure, which has a reported link with chromosome breakage and reduced repair efficiency (Pero et al., 1976). Clearly much more information is required before any firm conclusions are drawn about this sort of association.

5. Aneuploidy and Somatic Mutation. In the present study of ageing, the numbers of chromosome breaks do not show a marked age-related change, and while they do increase in females the common exposure to X-rays and environmental

mutagens is a possible cause for this. There is thus no clear evidence for somatic mutation expressed as increased chromosomal fragility, although the aneuploid changes may result from mutations in processes controlling cell division or some other factor.

v. Somatic Mutation, Repair and Ageing

If somatic mutation is the main cause of ageing, it is important to know why the mutations arise and how their frequency is controlled genetically in order to control longevity. The chromosome instability syndromes described in Part I (Ataxia Telangiectasia, Fanconi's Anaemia and Bloom's syndrome) are inherited as simple autosomal recessives and their obvious somatic mutation is associated with an increased cancer incidence and probably defective repair of damaged DNA. Lack of efficient repair might well lead to accumulation of somatic mutations in ageing and there is evidence that blood cells from older individuals are more susceptible to chemical mutagenesis (Bochkov and Kuleshov, 1971). Hart and Setlow (1974) have studied UV-induced repair synthesis in a number of species and have found a possible correlation between the amount of 'unscheduled DNA synthesis' in fibroblasts and the species lifespan, although this is not a simple relationship since Xeroderma Pigmentosum patients whose excision repair can be at a lower level than that seen in the mouse, still live a good deal longer than mice! There is also evidence that unscheduled DNA synthesis is reduced in older individuals where a 25% reduction in this repair synthesis has been found in 60-94 year olds compared with

a 13-59 year age group (Lambert and Ringborg, 1976). DNA strand rejoining after gamma irradiation is reduced in another premature ageing syndrome known as Progeria (Epstein et al., 1973) and in vitro senescence of human fibroblasts is also associated with a reduction in excision of thymine bases damaged by gamma rays (Mattern and Cerutti, 1975). These changes are not associated with any alteration in the frequency of BrdU detected SCE (Part I, Chapter Four).

One candidate for a role as a somatic mutagen has been proposed by Baltimore (1974), namely the enzyme terminal deoxynucleotidyl transferase. This enzyme, which adds deoxyribonucleotides on to DNA primers without the template essential for other DNA polymerases, had been found only in calf thymus and was thought to occur in no other tissue until Baltimore and his group identified it in lymphoblasts from patients with acute lymphoblastoid leukaemia. The enzyme could act as a somatic mutagen as it could diversify the molecules that give immunological specificity to T cells, the thymus processed cells important in graft rejection and in cell recognition.

The many publications of Sir Macfarlane Burnet (see, for example, Burnet, 1974) have discussed widely somatic mutation, repair of genetic damage and the idea of immune surveillance (Burnet, 1970), where unusual cells are normally recognised as they arise and removed by the immune system; but if this mechanism breaks down 'forbidden clones' of cells may be able to proliferate as malignant growths. There is

a wide variety of evidence for alterations and deteriorations in the effectiveness of the immune system with age (see Walford, 1969) and the impression that blood samples from older subjects usually produce fewer dividing cells has been borne out by the evidence for the reduction with age of the response to the mitogen PHA, routinely used in chromosome studies (Pisciotta et al., 1967; Conard et al., 1971; Hallgren et al., 1973).

There is evidence that the proportion of T cells declines with age (see, for example, Smith et al., 1974; Augener et al., 1974), an observation that may be linked with physiological changes in the thymus gland itself (Simpson et al., 1975); but it is interesting that the studies using BrdU, described in Part I, showed that despite the lower mitotic index, the proportions of cells in their first, second and third in vitro mitosis in three-day cultures were not greatly altered in old people, so that cells that do respond in our PHA-stimulated cultures grow just as quickly as cells from younger donors. The reduction in numbers of T cells may be associated with the increased risk of cancer in older people (Doll, 1968) if they are involved in suppression of potentially malignant cells as suggested by the theory of immune surveillance.

## vi. Conclusions

In conclusion, studies using G-banding have confirmed that there is an age-associated increase in aneuploidy with characteristic differences between the sexes. A great deal of hypodiploidy in females involves loss of an X chromosome,

and a marked increase in the frequency of aneuploid cells does not occur until the late 50's and early 60's in either sex. One mechanism of X chromosome mitotic non-disjunction seems to be premature centromere division. A slight increase in the numbers of males showing a very low proportion of 45X-Y cells (usually 1% or less) was observed. The use of G-banding has allowed us to analyse in more detail the involvements of each chromosome in aneuploidy, but it does not greatly enhance the efficiency of detection of chromosome abnormalities since the results are very similar to data from earlier studies using conventional orcein staining. C- and G-banding have on the other hand been useful in establishing that the 'medium fragment' in cells from older women is a prematurely divided X chromosome and in identifying the 'small acrocentric chromsomes' lost in males. Further studies in females would be of interest to investigate whether the X chromosome loss is random or involves preferentially the active or inactive homologue.

The present study has illustrated forcibly the large individual variations and sampling variations in proportions of abnormal cells, even when techniques were standardised as far as possible and the analysis was carried out by one observer using one set of criteria. It is also evident that in spite of this, the overall picture gained from several studies is similar, but that many other factors besides the age of the subject contribute to the final proportion of observed aberrant cells. The non-disjunction may reflect a mutation in the normal control mechanisms of

mitosis, or changes in membrane fragility or other cellular properties, and may therefore be evidence for either somatic mutation or error accumulation, or both these processes together.

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# APPENDICES.

I. Galloway, S.M., and Evans, H.J., 1975.
Asymmetrical C-bands and satellite DNA in man.
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II. Galloway, S.M., and Evans, H.J., 1975.
Sister chromatid exchange in human chromosomes from normal individuals and patients with ataxia telangiectasia.
Cytogenet. Cell Genet. 15 17-29

## Asymmetrical C-bands and satellite DNA in man

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*Summary.* After one replication period in the presence of BUdR, lateral asymmetry of staining with the FPG technique is observed within the major C-bands of human chromosomes. This effect appears to be correlated with the locations of satellite DNAs, and may be partly due to strand bias in thymidine content of the DNA.

The incorporation of 5-bromodeoxyuridine (BUdR) into chromosomal DNA has been shown, under appropriate conditions, to modify the staining properties of the substituted chromatin. In cells that have completed two rounds of replication in the presence of BUdR, one of each pair of sister chromatids is bifilarly substituted with BUdR and stains less intensely than its unifilarly substituted partner, with Giemsa [1], certain fluorescent dyes [2, 3, 4] or combinations of both fluorescence and Giemsa (FPG) [3]. Lin et al. [5] have recently noted a differential staining in mouse chromosomes that have undergone only one round of replication in BUdR, where, in contrast to the remainder of the chromosome, the centromeric heterochromatin (C-band) shows a lateral asymmetry of staining with the fluorescent dye Hoechst 33258. A similar asymmetry of staining was also noted in the C-band region of the human Y chromosome [6]. Since it is known that mouse satellite DNA is confined to the C-band regions [7], and

that the two strands of mouse satellite DNA contain markedly different amounts of thymidine (45% and 22% [8]), it was proposed that the difference in fluorescence between the C-bands of the two sister chromatids was due to an asymmetric incorporation of BUdR. The same explanation was suggested to account for the differential fluorescence of the human chromosome [6].

In studies on human lymphocyte chromosomes stained by the FPG technique [3] following *two* rounds of replication in BUdR, we have noted [9] that in a proportion of cells the C-band region of the Y chromosome stained faintly, showing poor contrast between chromatids. Such a localised absence of staining at the M2 mitosis might be expected in a proportion of chromosomes derived from M1 cells showing lateral asymmetry of these segments at the first mitosis.

We have recently shown [10, 11] that the human Y chromosome does contain highly repetitive A–T-rich DNA and hybridises with cRNAs from each of the four human satellite DNAs. These satellite DNA's are also found in the C-band regions of various autosomes, and in particular in chromosomes 1, 9 and 16 [11, 12] and their distribution throughout the complement has now been mapped in some detail [11]. We have therefore attempted here to identify those human chromosome regions which show differential staining after one round of replication in BUdR and compare their distributions with those of the satellite DNAs. We show that the differential staining (fluorescence) with Hoechst 33258 can be obtained in both mouse and human chromosomes in a more permanent fashion using the FPG technique, and our results suggest that a correlation exists between the location of sites containing human satellite DNAs and regions showing differential staining at M1.

### *Material and Methods*

The methods used for blood lymphocyte culture, slide preparation and staining with the FPG technique [3] have been described previously [9]. The cultures were incubated for 45 to 52 h at 37°C to obtain a majority of cells in their first in vitro metaphase, or 72 h to obtain M2 and M3 cells. 5'-Bromodeoxyuridine' (BUdR, Sigma) was present throughout, at concentrations of 10 to 160 $\mu$M. The resultant staining patterns varied from G-banding to uniform staining or a C-banded effect, and although this depended on the age of preparations and the length of light exposure after staining with Hoechst 33258, a spectrum of staining patterns could be found on a single slide.

Mouse A9 cells were grown in RPMI 1640 supplemented with glutamine and 15% foetal calf serum, with added 10 $\mu$M BUdR. Metaphase cells were harvested by shaking, treated with 0.075 M KCl for 10 min, resuspended in 3 : 1 methanol : glacial acetic acid fixative, and dropped onto slides. Cells in their first division after addition of BUdR (M1 cells) were obtained after about 24 h, and M2 and M3 cells by 50 h.

### *Results and Discussion*

We have confirmed the asymmetrical staining effect seen by Lin et al. [5] in the C-band regions of mouse chromosomes, staining with the Hoechst 33258 fluorochrome and with the more permanent FPG technique. Application of the FPG technique to human chromosomes, after one round of DNA synthesis in BUdR, revealed a similar, but less marked, asymmetry between chromatids in various C-band regions in the complement. The frequencies with which the asymmetry could be detected in the major C-bands, i.e. those on chromosomes 1, 9, 16 and the Y are shown in table 1. These results show that there is a clear difference in staining between chromatids in the distal parts of the long arms of the Y chromosome. The contrast between chromatids within the C-band region of chromosome 16 is more marked (fig. 1), and in clearly stained preparations 100% of these chromosomes show asymmetry. The reduced

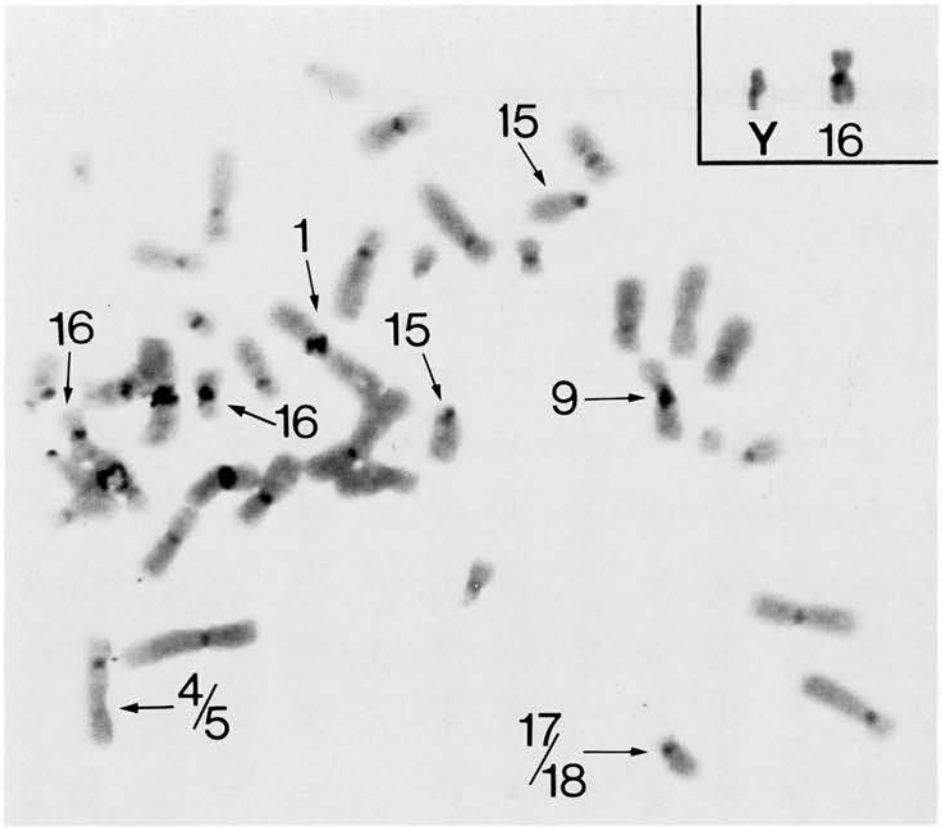


*Fig. 1.* A cell from a female, showing lateral asymmetry in the C-band regions of chromosomes 1 (compound), 15, 16, 17/18 and 4/5 (arrowed). The insert shows chromosomes 16 and Y from a male.

frequency of obvious asymmetry in our original observations on chromosome 1 is largely due to an interesting new polymorphism involving a differential staining within the C-band on each of the chromatids (see below). The large C-band region on chromosome 9 seldom showed unambiguous asymmetry. The FPG-stained region does not comprise the whole of the C-band, is often less intensely stained than the comparable areas of chromosomes 1 and 16, and has compound asymmetry that is less readily detectable and probably more complex than that of chromosome 1.

Staining asymmetry was also observed in the smaller C-band regions (and satellites) in other chromosomes in the complement and in particular in a D group chromosome. Where adequate G-banding was present,

Table 1. *Proportions of chromosomes showing asymmetrical C-bands at first mitosis*

| Chromosome no. | Total scored | Asymmetric *n* | Asymmetric (%) |
|---|---|---|---|
| 1 | 184 | 120 | 65 |
| 9 | 148 | 7 | <5 |
| 16 | 230 | 199 | 87 |
| Y | 60 | 54 | 90 |

Results pooled from 5 chromosomally normal individuals: 3 females and 2 males.

Table 2. *Comparative staining properties and satellite DNA content of human C-bands*

| | Chromosome | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 9 | 13 | 14 | 15 | 16 | 17 | 20 | 21 | 22 | Y |
| Asymmetrical staining with FPG | +++ | ± | − | − | ++ | +++ | + | + | + | + | +++ |
| C-banding | +++ | +++ | + | + | + | +++ | + | + | + | + | +++ |
| G-11 | + | +++ | + | + | + | − | + | + | + | + | ++ |
| Satellite DNA (11) | | | | | | | | | | | |
| Sat. I | | +++ | + | + | ++ | | | | + | ++ | +++ |
| Sat. II | +++ | +++ | | | ++ | ++ | + | | + | + | +++ |
| Sat. III | | +++ | + | + | ++ | | | + | + | ++ | +++ |
| Sat. IV | ++ | +++ | + | + | ++ | | + | + | + | ++ | +++ |

the chromosomes involved were identified as 15, 17, 20, 21 and 22. Lateral asymmetry was also observed in a B group chromosome which could not be unambiguously identified. In a few cells asymmetry of the satellites of chromosome 22 was evident, although the C-band was symmetrically stained.

The areas of asymmetrical staining in the human genome all contain satellite DNAs [11] (see table 2), and are all located within regions that stain positively with C-band techniques and, in general, with Giemsa at high pH (G-11 [13]). A detailed comparison of the distribution of these properties is given in table 2. Although there is a relationship between these four parameters, particularly demonstrated in the Y chromosome, there are some obvious discrepancies. The two most significant are the apparent rarity of clear asymmetry within the C-band of chromosome 9, which contains large amounts of all four satellite DNAs and is strikingly G-11 positive, and the marked asymmetry in chromosome 16, which contains significant amounts of satellite II only, and is G-11 negative. Indeed, the best correlation appears to exist between C-band asymmetry and the presence of DNA satellite II.

The four highly repetitive satellite DNAs in man all contain a higher proportion of A-T bases than main band DNA. However, the strand bias in thymidine content is not yet known for satellites III and IV, is small (4%) for satellite II DNA (30% and 26% in the heavy and light strands respectively, A. Mitchell, personal communication) and is about 10% (42% to 32%) for satellite I DNA [14]. Despite this small difference in total content of thymidine between strands of the satellite DNA in chromosome 16, it is probable that even slight changes in DNA–protein interaction following BUdR incorporation may be sufficient to alter chromatin staining properties quite markedly, and it has been shown that BUdR-substituted DNA has an increased affinity for proteins [15]. It is possible therefore that the original explanation proferred to account for the lateral asymmetry in staining of the C-band region of mouse chromosomes, i.e. that it is a result of a strand bias in BUdR incorporation [5], may also apply to the asymmetries noted in the human complement. However, this does not account for the apparent lower frequency of asymmetry in chromosomes 1 and 9 relative to 16 or Y.

In independent studies by Angell & Jacobs [16] (personal communication) using a similar staining technique on chromosomes unifilarly substituted with BUdR, it was noted that in some individuals

Table 3. *Proportion of chromosomes at second division (M2) showing asymmetrical C-bands*

| | Total scored | Asymmetrical *n* | Observed (%) | Expected (%) |
|---|---|---|---|---|
| Chromosome no. 1 | | | | |
| One homologue compound (4 individuals) | 70 | 55 | 78.6 | 75 |
| Both homologues compound (2 individuals) | 142 | 130 | 91.6 | 100 |
| Chromosome 16 (6 individuals) | 218 | 184 | 84.4 | 50 |

chromosome 1 showed a compound asymmetry in which both chromatids were stained within the C-band region, but with the stained dots at different loci. This prompted us to carry out further studies on cells from 22 different individuals and these confirm the presence of such compound asymmetry in chromosome 1 (fig. 1) in which one or more dark dots on each chromatid are located directly opposite pale staining regions on the sister chromatid. The particular pattern for a given number 1 chromosome is constant within the individual. In 9 of these individuals compound asymmetry was evident in one homologue and the remainder showed this in both, so that this is yet another polymorphic feature in the human chromosome complement. Our more detailed studies on chromosome 9 revealed the presence of a similar, but less clearly defined, compound asymmetry and this complex staining effect accounts for our original low estimate of the frequency of asymmetry in both chromosomes 1 and 9. One possible explanation for compound asymmetry is that it reflects inversions within the repetitive DNA's of the C-band where polarity of the polynucleotide chains is maintained by means of a switch of a segment of a thymidine-rich strand (giving pale staining after BUdR substitution) from one chain to the other. Such inversion switches could occur in mitotic or meiotic cells, but they must be of relatively rare occurrence for otherwise C-band asymmetry would not be observed. It is interesting that we have been unable to detect compound asymmetry in the large C-band region of the Y chromosome.

Segregation of chromatids of asymmetrically stained chromosomes following a second replication in the presence of BUdR, should result in M2 daughter chromosomes of which half have an asymmetrical C-band and half a completely pale C-band region. At M3 one quarter of the chromosomes would be expected to show C-band asymmetry. The mouse chromosomes do fit this distribution, and the human Y also conforms although a double pale tip on a small Y chromosome is not always easily detected. However, chromosome 1 and 16 do not follow the same pattern at M2 and M3, as the dark FPG-C-bands seem to be over-represented. In chromosome 1 this is the result of composite staining, and the distributions of C-banded chromatids at M2 and M3 follow those expected in individuals having compound asymmetry of one or both homologues (see table 3). In chromosome 16 the excess of chromatids with C-bands at M2 is the result of the relatively darker staining of the M1 (unifilarly substituted) 'pale' region relative to the M2 (bifilarly substituted), pale segment.

Since BUdR-substituted DNA in solution

quenches fluorescence of Hoechst 33258, it has been proposed [6] that the differential fluorescence is due to a greater degree of quenching by the bifilarly-substituted chromatin. The differentiation seen after Giemsa staining implicates a more complex reaction, possibly involving DNA–protein interactions, and the asymmetry seen here may reflect variations in content of not only satellite DNAs, but of other repetitious types of DNA known to occupy the C-band regions [17].

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# Sister chromatid exchange in human chromosomes from normal individuals and patients with ataxia telangiectasia

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*Abstract*

A new fluorescence plus Giemsa staining technique now makes the detection of sister-chromatid exchange (SCE) a relatively easy matter in cells containing 5-BrdU-substituted DNA. The technique has been applied to human cells to examine the distribution of SCE between different people and between and within different chromosomes. The results show: (1) That there were no large differences in the incidence of SCE between blood leukocyte chromosomes from male and female adults and newborn, and that similar frequencies were found in cells from two patients with ataxia telangiectasia which, nevertheless, showed the typical increases in chromosomal aberrations. (2) The distribution of SCE between chromosomes in the complement was found to be proportional to chromosome length, although the smaller chromosomes were under-represented, but not significantly so. (3) The distribution of SCE within chromosomes was nonrandom, with a deficiency in the centromeric and an excess in the mid-arm regions. There was no evidence for an excess of SCE in chromosome regions rich in AT DNA sequences. (4) The frequency of SCE is to some extent dependent on 5-BrdU concentration, but the influence of concentration is minimal within the range of from 1 to 160 $\mu$M. Human cells exposed over two cell cycles at these higher BrdU levels have around 14 SCE per cell–a frequency virtually identical with that observed in cultured cells from the Chinese hamster, wallaby, and rat kangaroo.

A number of rare inherited disease states in man are associated with markedly raised frequencies of chromosome aberrations in peripheral blood lymphocytes and cultured fibroblasts. The best known of these "chromosome instability syndromes" are Fanconi's anemia (SCHROEDER

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et al., 1964; GERMAN and CRIPPA, 1966), Bloom's syndrome (GERMAN et al., 1965; GERMAN, 1969) and ataxia telangiectasia (Louis-Bar syndrome) (HECHT et al., 1966; GROPP and FLATZ 1967), and all three are inherited as autosomal recessives. The aberrations are of the chromatid and chromosome type, and there are a variety of reasons for suspecting that increased frequencies of exchange between subunits of sister chromatids (sister-chromatid exchange, or SCE) might also exist. Until recently, SCE's have only been detectable by autoradiography since, unlike chromosomal aberrations, they involve no apparent change in morphology. For this reason we have only minimal information on the incidence and distribution of SCE in human chromosomes in cells from normal individuals and none whatsoever on the chromosomes of individuals with these rare heritable genomic instabilities.

Recently, LATT (1973) has shown that sister chromatids in human lymphocyte cells fluoresce differentially when stained with Hoechst 33258 if the cells are allowed to undergo two rounds of replication in the presence of 5-bromodeoxyuridine (5-BrdU) prior to fixation and staining. With this technique, SCE could be observed without recourse to autoradiography, although the differential dye binding/fluorescence with Hoechst 33258 is extremely photo-unstable and subject to rapid image fading. In our laboratory, the work of PERRY and WOLFF (1974) with Chinese hamster cells has now shown that subsequent Giemsa staining of 5-BrdU-substituted and Hoechst 33258-stained chromosomes yields nonfading preparations in which sister chromatids are very clearly and permanently differentially stained. We have now applied the PERRY and WOLFF fluorescence plus Giemsa (FPG) technique to human chromosomes and in this report describe the incidence and distribution of SCE within and between chromosomes in cultured blood lymphocytes from various groups of normal individuals and from two patients with ataxia telangiectasia.

## *Materials and methods*

The normal individuals included seven healthy adults, five males and two females (groups I and IV) encompassing an age range of from 26 to 35 years, and six newborn babies, four males and two females (groups II and V). The patients with ataxia telangiectasia (group III) were two brothers aged 9 and 11 years.

Cord blood or "heel-prick" samples were used for blood cultures from the babies and venous blood from the patients and adults. Blood samples from the patients were kindly provided by Dr. J.A. RAEBURN. Whole-blood microcultures

(HUNGERFORD, 1965) were incubated at 37° C using McCoy's or Eagle's medium supplemented with 18 % fetal calf serum and glutamine with PHA, penicillin, and streptomycin. 5-BrdU was added to each culture to give a concentration of 50 μg/ml (160 μM), and the cultures were protected from light to avoid SCE due to photolysis of 5-BrdU-substituted DNA (IKUSHIMA and WOLFF, 1974). All cultures were treated with Colcemid (0.001 %) for the final 2 1/2 h of incubation and harvested at 69–73 h, when a significant proportion (25–60 %) of the mitotic cells had undergone two rounds of DNA synthesis in vitro. Standard air-dried chromosome preparations were stained by the FPG technique (PERRY and WOLFF, 1974), using the fluorescent dye Hoechst 33258, followed by Giemsa staining.

## *Results and discussion*

The FPG technique is seen to be as effective on human as on Chinese hamster chromosomes, and a typical lymphocyte metaphase plate with the "Harlequin chromosomes" is shown in fig. 1. A striking differentiation between chromatids may also be seen, under fluorescence microscopy, after staining with acridine orange (5 min in 0.005 % acridine orange in Sorensen's buffer, pH 6.7). We find a less marked differentiation following staining with quinacrine dihydrochloride as compared with acridine

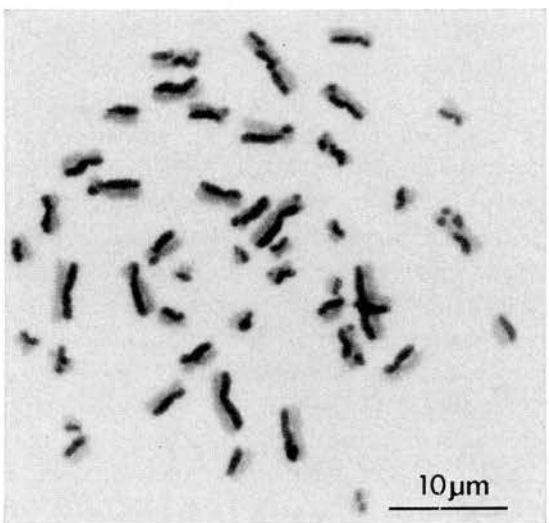


*Fig. 1.* Peripheral blood lymphocyte metaphase stained by the FPG technique showing the differentiation between chromatids in the "Harlequin-stained" chromosomes. The cell shows 21 sister-chromatid exchanges (SCE).

orange or Hoechst 33258, but, with quinacrine or acridine orange, contrast between chromatids increases during about 10–60 s exposure to ultraviolet light while viewing. Subsequent staining with Giemsa was successful when maximum fluorescence contrast had developed under ultraviolet light, but chromosomes which had faded after overexposure took up Giemsa only faintly even after 12 h in stain.

*Distribution of SCE between individuals and groups*

A total of 248 cells from the 15 individuals were scored for the frequency and distribution of SCE within the chromosome complement. In the majority of these cells it was not possible to identify unequivocally all chromosomes since the FPG technique does not give good G-banding profiles, particularly in those cells showing good "Harlequin-stained" chromosomes. For this reason, the chromosome complement was divided into 10 readily identifiable chromosomes or chromosome groups, viz., 1, 2, 3, B, C, D, 16, E, F, and G. Since exchange sites could not be easily related to G-bands, the distribution of SCE was mapped in relation to chromosome (arm) length and centromere position using the PARIS CONFERENCE (1971) map as a guide. In each chromosome a centromere region was delineated and each arm divided into one, two, or three equal-sized segments and the number of SCE's per segment determined in each cell (see fig. 3).

The over-all data are shown in table I, together with the partitioned data on G-group chromosomes to facilitate comparison between males and females. Because of uneven staining, chromosome overlaps, and chromosome loss due to cell breakage, all 46 chromosomes were not scored in all cells. There was no evidence for differential chromosome loss between individuals or groups, so that direct comparisons may be made from table I, which shows a range of mean frequency of SCE per chromosome of from 0.21 to 0.48. *T*-test comparisons between individuals within groups revealed no significant differences, except in the cases of the extremes in group I: between individuals 2 and 1 and between individuals 2 and 5 ($t = 4.8$ and 2.9 respectively, $P = <0.01$). Comparisons of the mean frequencies of SCE between groups also showed no significant difference, with the exception of comparisons made between the male babies (group II) and all other groups ($t = 3.26$, $P = <0.01$). The finding of a significant difference between a pair of individuals within a group is not surprising since this could be due a variety of factors. We are able to rule out the possibility that the difference is due to differential chromosome

*Table I.* Incidence of SCE in chromosomes from different individuals.

| Group | Individual | Total chromosomes | Total SCE | SCE per chromosome | No. of G-group chromosomes | SCE per G-group chromosomes |
|---|---|---|---|---|---|---|
| I Adults | 1 | 1284 | 271 | 0.211 | 140 | 0.057 |
| | 2 | 365 | 148 | 0.405 | 38 | 0.237 |
| | 3 | 369 | 107 | 0.289 | 33 | 0.061 |
| | 4 | 310 | 101 | 0.326 | 28 | 0.107 |
| | 5 | 329 | 75 | 0.228 | 35 | 0.086 |
| Total | 5 | 2657 | 702 | 0.264 | 274 | 0.091 |
| II Babies | 6 | 1308 | 628 | 0.480 | 132 | 0.144 |
| | 7 | 376 | 137 | 0.364 | 41 | 0.049 |
| | 8 | 326 | 147 | 0.451 | 33 | 0.152 |
| | 9 | 351 | 113 | 0.323 | 37 | 0.081 |
| Total | 4 | 2361 | 1025 | 0.434 | 243 | 0.119 |
| III Ataxia | 10 | 1157 | 322 | 0.286 | 109 | 0.046 |
| telangiectasia | 11 | 1140 | 277 | 0.244 | 103 | 0.087 |
| Total | 2 | 2297 | 599 | 0.265 | 212 | 0.066 |
| Male total | 11 | 7315 | 2326 | 0.318 | 729 | 0.093 |
| IV Adults | 12 | 1155 | 267 | 0.234 | 98 | 0.041 |
| | 13 | 701 | 259 | 0.369 | 56 | 0.089 |
| Total | 2 | 1856 | 526 | 0.286 | 154 | 0.058 |
| V Babies | 14 | 337 | 91 | 0.270 | 30 | 0.067 |
| | 15 | 355 | 113 | 0.318 | 30 | 0.133 |
| Total | 2 | 692 | 204 | 0.295 | 60 | 0.100 |
| Female total | 4 | 2548 | 730 | 0.286 | 214 | 0.070 |
| Total both sexes | 15 | 9863 | 3056 | 0.310 | 943 | 0.088 |

loss (note also that the SCE per G-group chromosome in individual 2 is significantly greater, at the 1 % level, than in individual 1 [see table I]) but cannot for instance exclude the possibility that it is a consequence of differences in uptake and incorporation of 5-BrdU. Although all cultures were maintained in the dark, it is still possible that at least a proportion, if not all (see below), of the SCE's observed are induced by the 5-BrdU

(IKUSHIMA and WOLFF, 1974; WOLFF and PERRY, 1974). Their incidence, therefore, may not be a true reflection of the inherent "spontaneous" instability of chromosomes within an individual. Nevertheless, it is to be expected that the frequency with which chromosomes are involved in SCE will be influenced by the genotype, since evidence from other organisms shows that intraspecific genomic differences exist for chiasma frequency at meiosis and for levels of mutagen-induced chromosome damage in somatic and germ cells (SEARLE et al., 1970). It is therefore quite likely that different people may show different frequencies of SCE as well as differences in the incidence of other "spontaneous" chromosomal abnormalities. What can be said from the present data is that, within the range of samples examined, there are no large differences in SCE frequency between different normal individuals or between different groups.

In regard to the ataxia telangiectasia patients, routine analysis of lymphocyte chromosomes revealed the characteristic higher incidence of chromosomal anomalies as compared with all controls (M. O'RIORDAN, unpublished data). It was suspected that this inherent increase in chromatid interchange in these cells might well be paralleled by an increase in the frequency of SCE, particularly if both types of exchange phenomena were manifestations of an underlying instability at the replication level. Our results (table I), however, suggest that this is not the case since the incidence of SCE in the cells from the ataxia telangiectasia patients is clearly not different from controls.

*Distribution of SCE between chromosomes*

5-BrdU substitutes for thymine in the DNA, so that its concentration should be high in those chromosome regions rich in AT sequences. There is evidence (EVANS et al., 1974) that the centromeric C-band regions on chromosomes 1, 9, and 16 and the brightly fluorescing C-band region on the Y chromosome contain AT-rich DNA. If the SCE's observed are indeed induced by 5-BrdU, then it might be expected that the distal region of the Y chromosome should show a higher frequency of SCE than comparable regions in the other G-group chromosomes. The data in table I show that the mean frequency of SCE per G-group chromosome is somewhat higher in males than in females, but this difference is not significant ($t = 0.8$, $P>0.4$). It was often difficult to distinguish the Y chromosomes in preparations processed by the FPG technique, but where this was possible, there appeared to be no difference in SCE frequency as compared with the G-group autosomes in the same cells. On detailed

analysis of the total data (see fig. 3), however, we find that there is an excess of SCE in the distal half of the long arm of the G-group chromosomes in males as compared with females. On the other hand, there is no evidence for an excess of SCE's in the C-band regions of chromosomes 1, 9, and 16. Similarly, it should be noted that the level of exchanges in males studied here does not appear to depend upon the size of the Y chromosome as identified with quinacrine, and the brothers with ataxia telangiectasia who had very large Y chromosomes equivalent in size to an E-group chromosome had a somewhat lower rate of SCE for the G group as compared with other male individuals.

The incidence of SCE per chromosome, or chromosome group, in relation to relative chromosome length (PARIS CONFERENCE, 1971) is shown in table II and fig. 2. From the figure it may be seen that the frequency with which a chromosome is involved in SCE appears to be a simple function of its length. Chromosome 16 and the E, F, and G

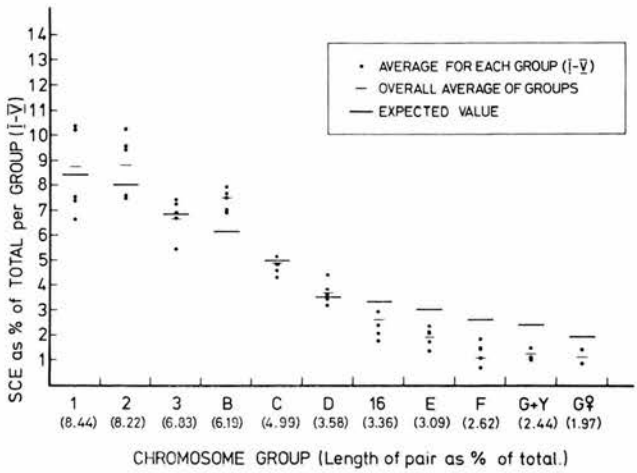


*Fig. 2.* Distribution of SCE between chromosomes as a percentage of the total SCE scored for each of the five groups of individuals. The abcissa shows chromosomes (groups) arranged in descending order of size, with relative lengths given in parenthesis. The points are the observed mean frequency of involvement of each chromosome for each group of individuals given as a percentage of the total SCE for each group. Thin horizontal bars are the mean values for each chromosome from the pooled data from all five groups of individuals; the thick bars are the calculated expected values if involvement in SCE is directly proportional to chromosome length.

*Table II.* Frequencies of SCE per chromosome or chromosome group.

| | Chromosome or group | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | B | C | D | 16 | E | F | G♂ | G♀ | G-group total | Over-all total |
| Total SCE | 275 | 277 | 217 | 472 | 1109 | 339 | 79 | 127 | 78 | 68 | 15 | 83 | 3056 |
| Total chromosomes | 465 | 442 | 427 | 876 | 3267 | 1275 | 429 | 875 | 864 | 729 | 214 | 943 | 9863 |
| SCE per chromosome | 0.59 | 0.63 | 0.51 | 0.54 | 0.34 | 0.27 | 0.18 | 0.15 | 0.09 | 0.09 | 0.07 | 0.09 | 0.31 |

groups are slightly under-represented, but these deviations are not significant. If SCE's are indeed distributed randomly throughout the chromosome complement, then the frequency with which a given chromosome is involved in 0, 1, 2, ..., *n* exchange events should conform to a Poisson distribution. The data on all chromosomes were subjected to a Poisson analysis, and all showed excellent fits to the expected distributions. We must conclude then that the exchanges are randomly distributed, a result in accord with earlier autoradiographic findings on tritium-labeled plant (TAYLOR, 1958; PEACOCK, 1963), animal (TAYLOR, 1965; GIBSON and PRESCOTT, 1974), and human (SMYTH and H.J. EVANS, unpublished data) chromosome complements.

Since the completion of our work, a paper by LATT (1974) has appeared showing a somewhat similar correlation between chromosome length and SCE incidence in human cells treated with 5-BrdU and Hoechst 33258, and LATT also finds that the smaller chromosomes contain fewer exchanges than expected.

*Distribution of SCE within chromosomes*

In contrast with the results on the distributions of SCE's between chromosomes, when we examine the distribution of SCE's within human chromosomes, marked deviations from expectation are to be found (fig. 3). In regard to chromosome 3, only 124 of the total of 217 SCE's could be unambiguously assigned to the long or short arm, and the two arms of the F-group chromosomes could be clearly distinguished in only 46 of the total of 78 cases. In general, it may be seen that there are relatively few SCE's at the centromeres and, in a number of chromosomes, the numbers of SCE in the proximal regions are often significantly below expectation, whereas the mid-regions contain an excess. Our attempts to relate exchange points to Q- or G-bands were not very successful, but LATT (1974) has recently reported that, at least in the case of chromosome 1, many of the SCE's appear to be located in the pale-staining or Q-negative bands. This nonrandom distribution pattern of SCE's within human chromosomes is similar in some respects to the patterns for chromosome- and chromatid-type aberrations induced in X-irradiated (HOLMBERG and JONASSON, 1973; SAVAGE et al., 1973) or nitrogen-mustard-treated (R. HOWELLS and H.J. EVANS, unpublished data) lymphocytes where the centromeric, C-band, and terminal regions of chromosome arms are involved less frequently than expected. The low frequency of SCE at the centromere was confirmed by studying cells in their third mitosis after 5-BrdU treatment

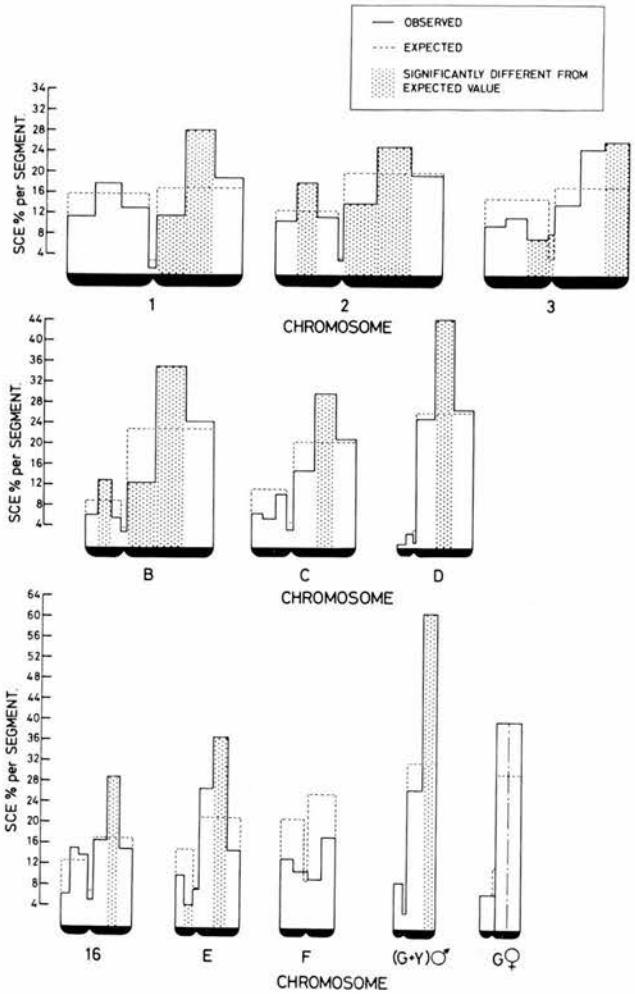


*Fig. 3.* SCE per segment as a percentage of the total SCE per chromosome. The continuous lines are the observed distributions and the dotted lines the expected uniform random distributions based on chromosome arm length. The shaded columns are segments containing an excess or deficiency of SCE which differ significantly from the expected value ($P<0.05$) by chi-square testing.

to avoid misclassifying twisted chromatids as SCE's. In these third-division cells, 195 exchanges were observed in 550 chromosomes, and only 10 of these ($\approx 5\,\%$) were located at the centromere. This is similar to a result obtained in wallaby chromosomes (GEARD, 1974) with tritium labeling

and autoradiography but contrasts with the earlier autoradiographic findings of others showing a high frequency (≈20 %) of SCE at the centromeres in cells from the rat kangaroo (GIBSON and PRESCOTT, 1974) and Chinese hamster (MARIN and PRESCOTT, 1964).

Studies on SCE in Chinese hamster ovary cells exposed in the dark to 5-BrdU and analyzed with the FPG technique have shown (WOLFF and PERRY, 1974) that the yield of exchanges increases with increasing concentration of analog within the range of from 0.3 μM to 1.0 μM. At higher concentrations (1–20 μM), the yield saturates at a level of around 12 SCE per cell over the two division cycles. A similar plateau in SCE frequency is found in rat kangaroo cells exposed to increasing doses of tritium (GIBSON and PRESCOTT, 1972), where the yield is maximal at a level of around 10 SCE per cell over two division cycles. A frequency of 14 SCE per cell over two cell cycles is also evident in published data on wallaby chromosomes (GEARD, 1974). The present data on human chromosomes were obtained on cells exposed to 5-BrdU at 160 μM (well above saturation level) and give an SCE frequency of 14 per cell (range, 2–25 per cell) over two division cycles, and LATT's (1974) recent results give a frequency of 13 SCE per cell after two cycles in 20 μM 5-BrdU. The similarity between the results of these different experiments on different cell types is presumably a reflection of the similarity in DNA content per cell between these species; why the yields of SCE should saturate at these relatively low frequencies, i.e., around 1 SCE per μg of DNA, is, however, a mystery. What is clear is that, at these relatively high levels of 5-BrdU, many of the SCE's must be induced by the analog and presumably reflect exchanges initiated by single-strand breaks induced in the DNA; i.e., they reflect a DNA repair process that results in an exchange of DNA subunits. In this context, it would certainly be of interest to examine the incidence of SCE's in cells from patients with other inherited chromosomal instability syndromes and to measure the incidence of SCE in cells and individuals exposed to known mutagens.[1]

[1] Since this paper was submitted, a report by CHAGANTI et al. (CHAGANTI, S.K.; SCHONBERG, S. and GERMAN, J.: A manifold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. Proc. natn. Acad. Sci. USA *71:* 4508–4512 [1974]) has been published which describes the very interesting finding of a dramatic (12-fold) increase in SCE in lymphocyte chromosomes of patients with Bloom's syndrome. These authors also report that the frequency of SCE in three cells from a patient with ataxia telangiectasia did not differ from controls, an observation in line with our own findings. It would appear that further studies on the incidence of SCE

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in cells from patients with inherent chromosomal instability, and on their frequency following exposure of the cells to chemical mutagens, should certainly prove rewarding.

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APPENDIX III a.

FEMALE HYPODIPLOID CELLS AS % TOTAL CELLS.

| | 0 | 14 | 20-24 | 25-29 | 30-34 | 35-39 | 40-44 | 45-49 | 50-54 | 55-59 | 60-64 | 65-69 | 70-74 | 75-79 | 80-84 | 85-89 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total E | 1.33 | 1.0 | 0.67 | 1.0 | 4.29 | 2.0 | 5.33 | 6.0 | 2.73 | 4.25 | 4.0 | 10.77 | 13 | 5.96 | 19.0 | 4.0 |
| Hypo. L | 3.43 | 0 | 3.2 | 2.0 | 4.67 | 2.86 | 5.1 | 4.0 | 3.55 | 3.3 | 4.12 | 4.93 | 9.45 | 6.0 | 19.0 | 6.0 |
| E+L | 2.1 | 0.53 | 1.97 | 1.5 | 4.5 | 2.5 | 5.17 | 4.62 | 3.1 | 3.3 | 4.12 | 6.19 | 8.41 | 5.98 | 19.0 | 5.0 |
| 45-X E | 0 | 0 | 0 | 0 | 0.29 | 0.4 | 1.33 | 1.0 | 0.18 | 1.42 | 2.0 | 5.36 | 2.45 | 0 | 7 | 0 |
| L | 0 | 0 | 0 | 0 | 1.3 | 0.57 | 1.78 | 0.67 | 0.89 | 0.79 | 0.72 | 1.97 | 2.2 | 2.0 | 14.0 | 0 |
| E+L | 0 | 0 | 0 | 0 | 0.88 | 0.5 | 1.67 | 0.77 | 0.5 | 0.99 | 0.78 | 2.71 | 2.3 | 1.0 | 10.5 | 0 |
| 45- E | 0.67 | 0 | 0 | 0 | 0.57 | 0.4 | 1.33 | 2.0 | 0.55 | 0.28 | 2.0 | 2.38 | 0.67 | 3.0 | 3.0 | 0 |
| (6-12) L | 0.57 | 0 | 0.65 | 0 | 0.89 | 0 | 0.44 | 0.67 | 0 | 0.53 | 0.21 | 0.66 | 1.82 | 0.67 | 0 | 0 |
| E+L | 0.63 | 0 | 0.33 | 0 | 0.75 | 0.17 | 0.67 | 1.08 | 0.3 | 0.45 | 0.2 | 1.03 | 1.3 | 1.0 | 1.5 | 0 |
| 45-all E | 0.33 | 0 | 0 | 0 | 1.43 | 0 | 1.33 | 2.0 | 1.27 | 1.7 | 0 | 1.19 | 2.0 | 0.67 | 1.0 | 3.0 |
| other L | 1.14 | 0 | 1.29 | 0 | 1.56 | 0.57 | 1.11 | 1.33 | 0.67 | 1.19 | 1.65 | 1.15 | 2.0 | 2.67 | 3.0 | 2.0 |
| E+L | 0.63 | 0 | 0.66 | 0 | 1.5 | 0.33 | 1.17 | 1.54 | 1.0 | 1.35 | 1.57 | 1.16 | 2.0 | 1.67 | 2.0 | 2.5 |
| 45- E+L 21/22 | 0 | 0 | 0 | 0 | 0.5 | 0 | 0 | 0.62 | 0.2 | 0.36 | 0.39 | 0.52 | 0.5 | 0.33 | 0.5 | 0.5 |

## APPENDIX III b.

## FEMALE HYPERDIPLOID CELLS AS % TOTAL CELLS.

| | | 0 | 14 | 20-24 | 25-29 | 30-34 | 35-39 | 40-44 | 45-49 | 50-54 | 55-59 | 60-64 | 65-69 | 70-74 | 75-79 | 80-84 | 85-89 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Total hyper. | E | 0 | 0 | o | 1.0 | 0 | 1.2 | 2.0 | 0.5 | 0.55 | 1.13 | 4.0 | 1.31 | 1.78 | 0.66 | 4.0 | 1.0 |
| | L | 0.57 | 0 | 0.65 | 0 | 0.89 | 0.29 | 0.67 | 0.89 | 1.55 | 0.92 | 1.13 | 1.97 | 1.82 | 0.67 | 2.0 | 0 |
| | E+L | 0.21 | 0 | 0.33 | 0.5 | 0.5 | 0.67 | 1.0 | 0.77 | 1.0 | 0.91 | 1.27 | 1.8 | 1.8 | 0.66 | 3.0 | 0.5 |
| 47+X | E | 0 | 0 | 0 | 1.0 | 0 | 0 | 0 | 0.5 | 0.36 | 0.85 | 4.0 | 1.19 | 0.67 | 0 | 2.0 | 1.0 |
| | L | 0 | 0 | 0 | 0 | 0.44 | 0.29 | 0.22 | 0.67 | 0.44 | 0.66 | 0.41 | 1.15 | 1.27 | 0.67 | 2.0 | 0 |
| | E+L | 0 | 0 | 0 | 0.5 | 0.25 | 0.33 | 0.17 | 0.62 | 0.4 | 0.72 | 0.59 | 1.16 | 1.0 | 0.33 | 2.0 | 0.5 |
| 47+ (6-12) | E | 0 | 0 | 0 | 1.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.6 | 0 | 0 | 0 | 1.0 |
| | L | 0 | 0 | 0 | 0 | 0 | 0 | 0.22 | 0 | 0 | 0.26 | 0.21 | 0.49 | 0.18 | 0.67 | 0 | 0 |
| | E+L | 0 | 0 | 0 | 0.5 | 0 | 0 | 0.17 | 0 | 0 | 0.18 | 0.2 | 0.52 | 0.1 | 0.33 | 0 | 0.5 |
| 47+ other | E | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.36 | 0.28 | 0 | 0.6 | 0.22 | 0 | 0 | 0 |
| | L | 0.57 | 0 | 0 | 0 | 0 | 1.0 | 0 | 0.67 | 0 | 0 | 0.1 | 0 | 0.36 | 0 | 0 | 0 |
| | E+L | 0.21 | 0 | 0 | 0 | 0 | 0.17 | 0 | 0.46 | 0.2 | 0.09 | 0.1 | 0.13 | 0.3 | 0 | 0 | 0 |

## APPENDIX III c.

## FEMALE B, CU, and CS CELLS AS % TOTAL CELLS.

| | | 0 | 14 | 20-24 | 25-29 | 30-34 | 35-39 | 40-44 | 45-49 | 50-54 | 55-59 | 60-64 | 65-69 | 70-74 | 75-79 | 80-84 | 85-89 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| "B" | E | 0 | 6.0 | 1.3 | 0 | 2.0 | 1.6 | 1.33 | 3.5 | 2.36 | 0.88 | 0 | 3.57 | 1.1 | 0 | 6.0 | 5.0 |
| | L | 1.14 | 1.4 | 0.65 | 0 | 1.1 | 0.86 | 1.1 | 1.78 | 1.77 | 0.26 | 0.41 | 1.48 | 1.27 | 2.0 | 0 | 1.0 |
| | E+L | 0.42 | 3.72 | 0.98 | 0 | 1.5 | 1.17 | 1.17 | 2.31 | 2.1 | 0.41 | 0.39 | 1.93 | 1.2 | 0.99 | 3.0 | 3.0 |
| "Cu" X fr. | E | 0 | 0 | 0 | 0 | 4.0 | 1.6 | 2.0 | 0.5 | 2.0 | 1.7 | 0 | 2.38 | 4.01 | 5.3 | 5 | 2.0 |
| | L | 0 | 0 | 0 | 0 | 0.4 | 0.29 | 1.33 | 0.44 | 0.89 | 0.8 | 1.75 | 2.96 | 1.09 | 2.0 | 3.0 | 1.0 |
| | E+L | 0 | 0 | 0 | 0 | 2.0 | 0.83 | 1.5 | 0.46 | 1.5 | 1.2 | 1.67 | 2.96 | 2.4 | 3.65 | 4.0 | 1.5 |
| "Cu" no fr. | E | 0.33 | 0 | 0 | 0 | 0.86 | 0 | 0 | 0.5 | 0.36 | 0.28 | 0 | 0 | 0.67 | 0 | 1.0 | 0 |
| | L | 0 | 0 | 0.65 | 1.0 | 0.67 | 0.86 | 0.22 | 1.33 | 0.44 | 0.53 | 0.21 | 0.99 | 1.64 | 0.67 | 1.0 | 0 |
| | E+L | 0.21 | 0 | 0.33 | 0.5 | 0.75 | 0.5 | 0.17 | 1.08 | 0.4 | 0.45 | 0.2 | 0.77 | 1.2 | 0.33 | 0.5 | 0 |
| "Cs" | E | 0 | 0 | 0 | 0 | 0 | 0 | 1.33 | 0 | 0.36 | 0.57 | 0 | 0.6 | 0.67 | 1.32 | 0 | 1.0 |
| | L | 0.57 | 0 | 0 | 0 | 0 | 0.29 | 0.89 | 0.66 | 0.89 | 0.26 | 0.93 | 0.16 | 0.73 | 0 | 0 | 0 |
| | E+L | 0.21 | 0 | 0 | 0 | 0 | 0.17 | 1.0 | 0.46 | 0.59 | 0.33 | 0.88 | 0.26 | 0.7 | 0.66 | 0 | 0.5 |

## APPENDIX IIId.

## MALE ANEUPLOID CELLS AS % TOTAL CELLS.

| | 0 | 14 | 20-24 | 25-29 | 30-34 | 35-39 | 40-44 | 45-49 | 50-54 | 55-59 | 60-64 | 65-69 | 70-74 | 75-79 | 80-84 | 85-89 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hypo. E | 0.8 | 0 | 4.0 | 1.0 | 4.5 | 0.7 | 2.0 | 3.33 | o.12 | 3.2 | 8.0 | - | 6.0 | 6.0 | 7.33 | - |
| L | 1.49 | 0 | 1.5 | 1.74 | 2.55 | 3.7 | 2.86 | 3.42 | 4.0 | 5.33 | 8.0 | - | 8.0 | 2.0 | 6.0 | 0.77 |
| E+L | 1.11 | 0 | 2.6 | 1.55 | 3.07 | 2.8 | 2.52 | 3.39 | 2.73 | 4.36 | 8.0 | - | 7.0 | 4.0 | 7.0 | 0.77 |
| 45-Y | 0 | 0 | 0 | 0.26 | 0.13 | 0.6 | 0.12 | 0.38 | 0 | 0.18 | 1.0 | - | 1.0 | 0 | 1.0 | 0 |
| 45-21/22 | 0 | 0 | 0.29 | 0 | 0.13 | 0.2 | 0.24 | 0 | 0.18 | 0.91 | 0.5 | - | 2.0 | 1.0 | 1.0 | 0 |
| Hyper. E | 0.4 | 0 | 0 | 0 | 0.5 | 0 | 0 | 0.33 | 0 | 0 | 0 | - | 0 | 2.0 | 0 | - |
| L | 0 | 0 | 0 | 0 | 0.36 | 0.86 | 0.22 | 0.4 | 1.33 | 0.33 | 0 | - | 0 | 0 | 0 | 0.77 |
| E+L | 0.22 | 0 | 0 | 0 | 0.4 | 0.6 | 0.13 | 0.38 | 0.73 | 0.18 | 0 | - | 0 | 1.0 | 0 | 0.77 |

## APPENDIX III e.

### MALE B, CU AND CS CELLS AS % TOTAL CELLS.

| | | 0 | 14 | 20-24 | 25-29 | 30-34 | 35-39 | 40-44 | 45-49 | 50-54 | 55-59 | 60-64 | 65-69 | 70-74 | 75-79 | 80-84 | 85-89 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| "B" | E | 0 | 0 | 0 | 0 | 2.5 | 0 | 1.67 | 0.67 | 1.6 | 1.2 | 0 | - | 2.0 | 0 | 1.33 | - |
| | L | 0.99 | 0 | 0 | 0.35 | 1.27 | 0.86 | 0.88 | 1.61 | 1.0 | 0.33 | 1.0 | - | 2.0 | 0 | 2.0 | 0.77 |
| | E+L | 0.44 | 0 | 0 | 0.26 | 1.6 | 0.6 | 1.19 | 1.25 | 1.27 | 0.73 | 0.5 | - | 2.0 | 0 | 1.5 | 0.77 |
| "Cu" | E | 0 | 0 | 1.3 | 0 | 0.5 | 0 | 0 | 1.0 | 0.12 | 0.8 | 0 | - | 6.0 | 2.0 | 3.33 | - |
| | L | 0 | 0 | 0 | 0.35 | 1.45 | 0.86 | 1.1 | 0.6 | 2.0 | 0.33 | 0 | - | 2.0 | 0 | 2.0 | 0 |
| | E+L | 0 | 0 | 0.57 | 0.26 | 1.2 | 0.6 | 0.66 | 0.75 | 1.64 | 0.55 | 0 | - | 4.0 | 1.0 | 3.0 | 0 |
| "Cs" | E | 0 | 0 | 0 | 0 | 0 | 0 | 0.33 | 0.67 | 0.4 | 0.4 | 4.0 | - | 0 | 0 | 0.67 | - |
| | L | 0 | 0 | 0.5 | 0 | 0.36 | 0 | 0.22 | 0.2 | 0.67 | 0 | 4.0 | - | 0 | 2.0 | 0 | 0 |
| | E+L | 0 | 0 | 0.29 | 0 | 0.27 | 0 | 0.26 | 0.34 | 0.55 | 0.18 | 4.0 | - | 0. | 1.0 | 0.5 | 0 |

## APPENDIX IV.

### REGRESSION ANALYSIS: FEMALES. (Root y = a + bx).

| Cell Category | Culture Time | a | b | SE | t | p less than |
|---|---|---|---|---|---|---|
| (A+B) Hypodiploid. | | | | | | |
| 45 | E | .507 | .023 | .354 | 3.39 | .01 |
| | L | .425 | .024 | .389 | 3.43 | .01 |
| 45-X | E | -.073 | .018 | .352 | 3.43 | .05 |
| | L | .266 | .015 | .447 | 1.86 | - |
| | E+L | .014 | .019 | .326 | 3.1 | .01 |
| 45-(6-12) | E | -.459 | .007 | .267 | 1.43 | - |
| | L | .025 | .007 | .318 | 1.2 | - |
| 45-other | E | .396 | .011 | .29 | 1.98 | .05 |
| | L | .565 | .010 | .192 | 2.99 | .05 |
| 45-any chrm ≠ X | E+L | .695 | .013 | .227 | 2.99 | .05 |
| All Hypodiploid. | E | .640 | .035 | .935 | 1.96 | - |
| | L | 1.219 | .017 | .366 | 2.59 | .05 |
| | E+L | 1.087 | .02 | .302 | 3.55 | .01 |
| (A+B)Hyperdiploid. | L | .421 | .010 | .229 | 2.47 | .05 |
| | E+L | .386 | .010 | .17 | 3.2 | .01 |
| All Hyperdiploid | E | .175 | .015 | .223 | 3.57 | .01 |
| | E+L | .418 | .011 | .185 | 3.08 | .01 |
| B + Cu + Cs | E | .936 | .024 | .387 | 3.22 | .01 |
| | L | 1.155 | .012 | .224 | 2.93 | .05 |
| Cu | E | .419 | .022 | .371 | 3.1 | .01 |
| | L | .328 | .018 | .169 | 5.93 | .01 |
| | E+L | .474 | .018 | .232 | 4.17 | .01 |
| Cu + X "fr". | E | .187 | .023 | .316 | 3.82 | .01 |
| | L | .328 | .018 | .169 | 5.93 | .01 |
| | E+L | .474 | 018 | .232 | 4.17 | .01 |
| Other Cu | E | .369 | .001 | .228 | .33 | - |
| | L | .48 | .005 | .258 | 1.16 | - |
| | E+L | .527 | .003 | .186 | 0.98 | - |
| Cs | E | -.113 | .013 | .216 | 3.0 | .01 |
| | L | .416 | .004 | .288 | .68 | - |
| | E+L | .171 | .008 | .201 | 2.2 | .05 |

## APPENDIX V.

### REGRESSION ANALYSIS: MALES. (Root y = a + bx).

| Cell Category | Culture Time | a | b | SE | t | p less than; |
|---|---|---|---|---|---|---|
| All Hypodiploid | E | .744 | .019 | .406 | 2.13 | .1 |
| | L | .113 | .014 | .294 | 2.11 | .1 |
| | E+L | .948 | .017 | .248 | 3.09 | .01 |
| 45-Y | E+L | .125 | .006 | .184 | 1.6 | - |
| All Hyperdiploid | E | No Regression | | | | |
| | L | .185 | .008 | .23 | 1.5 | - |
| | E+L | .359 | .002 | .191 | 1.88 | - |
| B + Cu + Cs | E+L | .724 | .015 | .237 | 2.47 | .05 |